In vitro and In vivo characterization of anti-biofilm activity of LpxC inhibitor alone, and in combination with different classes of antimicrobials

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

***IN VITRO* AND *IN VIVO* CHARACTERIZATION
OF ANTI-BIOFILM ACTIVITY OF LPXC INHIBITOR ALONE,
AND IN COMBINATION WITH DIFFERENT CLASSES OF
ANTIMICROBIALS**

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SINGAPORE CENTRE FOR ENVIRONMENTAL LIFE SCIENCES
ENGINEERING**

2017

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List of Abbreviations and Symbols

Terms	Abbreviation
<i>Pseudomonas aeruginosa</i>	<i>P.aeruginosa</i>
<i>Escherichia coli</i>	<i>E.coli</i>
Cystic Fibrosis	CF
Extracellular matrix	EPS
Extracellular deoxyribonucleic Acid	eDNA
Lipopolysaccharide	LPS
4-amino-4-deoxy-L-arabinose	LAra4N
3-deoxy-D-manno-erythronolonic acid	Kdo
Cytidine Triphosphate	CTP
National Institute of Health	NIH
World Health Organization	WHO
Extended spectrum β -lactamase	ESBL
UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase	LpxC

List of Publications

1. **Tan Jun Hou**, Celine Vidaillac, Joey Kuok Hoong Yam, Song Lin Chua, Michael Givskov, Liang Yang. 2017. *In vitro and in vivo efficacy of an LpxC inhibitor, CHIR-090, alone or combined with colistin against Pseudomonas aeruginosa biofilms*. Antimicrob Agents Chemother 61:e02223-16.

Summary

Biofilms are multicellular bacterial communities that are formed on a surface and encased in extracellular polymeric substance (EPS) made up of eDNA, proteins and exopolysaccharides. Due to their distinct - intrinsic, acquired and adaptive - antimicrobial resistance, biofilm formation is one of the main common causes of chronic infections and is becoming increasingly difficult to treat with existing antibiotics. Without anti-biofilm therapy that targets biofilm specifically, combination antimicrobial therapy remains one of the most effective strategies to eradicate biofilms.

CHIR-090 is a new class of antipseudomonal agent that inhibits LpxC enzyme, which in turn disrupts the formation of the lipopolysaccharides (LPS) anchoring protein, Lipid A. While CHIR-090 has been extensively characterised against planktonic bacteria, its anti-biofilm activity has yet been studied.

Understanding the stress responses and possible resistance mechanisms that arise during CHIR-090 treatment can provide useful information to help prolong the therapeutic longevity of the LpxC inhibiting compounds and the data could be used for the selection criteria for antimicrobial combinations. In this study, we applied RNA-sequencing (RNA-seq) analysis and experimental adaptive evolution assays to study the stress responses and development of resistance mechanisms by bacterial pathogen during

CHIR-090 treatment. We also evaluated the anti-biofilm activity of CHIR-090 alone, and in combination of other classes of antimicrobials using *in vitro* and *in vivo* methods such as antibiotic susceptibility testing, flow cell biofilms model and mouse implant biofilm model.

Our transcriptomic analyses showed upregulation of three gene clusters associated with CHIR-090 treatment: (i) *faoAB* operon with its *psrA* transcription regulator involved in fatty acid synthesis, (ii) *mexCD-OrpJ* that encodes for RND efflux pump and (iii) *phoPQ-oprH*, a two component transcription regulator. Meanwhile, experimental adaptive evolution assays identified six mutated genes from CHIR-090 resistant variants after 21 days of cultivation of *P. aeruginosa* strain in sub-inhibitory concentrations of CHIR-090. These mutated genes are classified into three categories based on their functions: (i) efflux pump (*mexR*), (ii) cell membrane and fatty acid synthesis (*murB*, *pyrG* and *fabF₁*) and (iii) motility (*morA* and *fliF*).

In vitro and *in vivo* evaluation of anti-biofilm activity of CHIR-090 demonstrated that the compound targeted the metabolically active subpopulation of *P. aeruginosa* biofilms, but administrated alone was ineffective in eradicating the entire biofilms. The combination of CHIR-090 and colistin displayed synergistic efficacy against colistin-susceptible and non-susceptible *P. aeruginosa* biofilms, while that combination of CHIR-090 and tobramycin was also synergistic against *P. aeruginosa* biofilms, albeit less effective than the CHIR-090-colistin combination.

In conclusion, this study provided evidence for the stress response and resistance mechanisms that *P. aeruginosa* developed during CHIR-090 treatment; it also demonstrated the therapeutic potential of CHIR-090 in combination with other antimicrobial agents against *P. aeruginosa* biofilms.

Chapter 1: Introduction

1.1 Biofilms and its implication

Bacterial cells can exist in two distinct lifestyles - planktonic and biofilms - depending on the physiological and environmental cues such as nutrient availability, cell density, osmotic stress or antimicrobial treatment. [1] Biofilms are multicellular bacterial communities that are formed on a surface and encased in an extracellular polymeric substance (EPS) made up of eDNA, proteins and exopolysacchrides. [2] The development of planktonic bacterial cells into biofilms occurs in several distinct stages (Figure 1.1). The first stage is the reversible attachment of the planktonic bacteria onto a surface, a process that can be reserved by polar flagellar activity. [3] The bacterial cells then aggregate longitudinally along the surface and develop into microcolonies, forming an irreversible attachment to the surface. These microcolonies further mature into macrocolonies that have thick bacterial cells embedded within the matrix. Lastly, the bacterial cells disperse from these mature macrocolonies and colonize other sites to form new biofilms. [4,5]

Figure 1.1: Life cycle of biofilm development. (a) Reversible attachment of planktonic bacteria to surface; (b) Irreversible attachment due to aggregation of bacteria cells; (c) Development of microcolonies; (d) Maturation of microcolonies into macrocolonies; (e) Dispersion of the bacterial cells from macrocolonies. Figure is adapted from [6] with permission.

Biofilms are fundamentally different from planktonic bacteria in their morphology, metabolisms and physiology. [7-9] They are highly resistant to antimicrobial treatments as compared to planktonic bacteria. [10-12] As a result, they are capable of causing biofilm-associated infections at multiple sites of the human body (Figure 1.2). Patients with biofilm-associated infections undergo longer treatment duration and, incur steeper medical cost. In US alone, the associated cost for the treatment of biofilm-associated infections had been estimated to exceed US\$1 billion. [13]

Biofilm-associated infections can be classified into device-related infections and tissue-related infections. Device-related infections are usually caused by biofilms originally

formed on implanted medical devices such as mechanical heart valves, urinary catheters, and endotracheal tube. [14] Pathogens may originate from the epithelial flora of patients and healthcare personnel, or from the environment, forming infectious biofilms on the surface of these implants, subsequently gaining access to organs or tissues post-implantation. [14] On the other hand, tissue-related infections are usually chronic and opportunistic in sterile locations of the body. Tissue-related infections include: chronic lung infections of cystic fibrosis (CF) patients, urinary tract infections, chronic wounds infections and periodontitis. [14] Biofilms formed on artificial and natural surface are vastly different in terms of their matrix compositions and physiological profiles. For example, *Staphylococcus aureus* forms compositionally unique biofilms made up of mostly fibrin and glyocalyx sheaths when it interacts with the host tissue protein (plasma). These substances help *S. aureus* to anchor the matrix to the infected tissue. [15]. Also, the characteristics of the artificial surface will have an effect on the rate and extent of pathogen colonization and biofilms generally develop faster on rougher and hydrophobic materials. [16]

Figure 1.2: Different sites for biofilms infection. Figure is adapted from [14] with permission.

Biofilm-associated infections can be caused by either a single bacterial species or a mixed-species microbial community. Bacterial interspecies interactions usually increase a biofilm's antimicrobial resistance. [17] The common pathogens involved in biofilm-associated infections include both Gram-positive and Gram-negative bacteria and fungi. [Figure 1.5] Among these pathogens, *P.aeruginosa* is considered one of the major contributors of biofilm-associated infections, affecting more than 2 million patients every year with an annual mortality rate of 90,000 lives. [18] About 30% of the CF infants

acquire *P.aeruginosa* strains from the environment. [19] This rate increases to 50% by the age of 3 years. *P.aeruginosa* mucoid variants with enhanced biofilm formation capacity have been reported in patients aged 3 to 16 years old. [20-22] Furthermore, *P.aeruginosa* can adapt to CF airways and persist as a lifelong ineradicable infection in almost 70% of adult CF patients. [23]

Table 1. Partial list of human infections involving biofilms.

Infection or disease	Common biofilm bacterial species
Dental caries	Acidogenic Gram-positive cocci (e.g. <i>Streptococcus</i>)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypable strains of <i>Haemophilus influenzae</i>
Musculoskeletal infections	Gram-positive cocci (e.g., <i>Staphylococci</i>)
Necrotizing fascitis	Group A <i>Streptococci</i>
Biliary tract infection	Enteric bacteria (eg., <i>Escherichia coli</i>)
Osteomyelitis	Various bacterial and fungal species – often mixed
Bacterial prostatitis	<i>E. coli</i> and other Gram-negative bacteria
Native valve endocarditis	<i>Viridans</i> Group <i>Streptococci</i>
Cystic fibrosis pneumonia	<i>P. aeruginosa</i> and <i>Burkholderia cepacia</i>
Melioidosis	<i>Pseudomonas pseudomallei</i>
Nosocomial infections	
ICU pneumonia	Gram-negative rods
Sutures	<i>Staphylococcus epidermidis</i> and <i>S. aureus</i>
Exit sites	<i>S. epidermidis</i> and <i>S. aureus</i>
Arteriovenous shunts	<i>S. epidermidis</i> and <i>S. aureus</i>
Scleral buckles	Gram-positive cocci
Contact lens	<i>P. aeruginosa</i> and Gram-positive cocci
Urinary catheter cystitis	<i>S. epidermidis</i> , <i>K. pneumoniae</i> , <i>E. faecalis</i> , <i>Proteus mirabilis</i>
Peritoneal dialysis (CAPD) peritonitis	A variety of bacteria and fungi
IUDs	<i>S. epidermidis</i> , <i>S. aureus</i> , <i>Corynebacterium</i> sp., <i>Micrococcus</i> sp., <i>Enterococcus</i> sp., <i>Candida albicans</i> , Group B <i>Streptococci</i> .
Endotracheal tubes	A variety of bacteria and fungi
Hickman catheters	<i>S. epidermidis</i> and <i>C. albicans</i>
Central venous catheters	<i>S. epidermidis</i> , <i>S. aureus</i> , <i>E. faecalis</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>C. albicans</i>
Mechanical heart valves	<i>Viridans streptococci</i> , <i>Enterococci</i>
Vascular grafts	Gram-positive cocci
Biliary stent blockage	A variety of enteric bacteria and fungi
Orthopedic devices	<i>Hemolytic streptococci</i> , <i>Enterococci</i> , <i>P. mirabilis</i> , <i>Bacteroides</i> sp., <i>P. aeruginosa</i> , <i>E. coli</i>
Pentile prostheses	<i>S. aureus</i> and <i>S. epidermidis</i>

Table 1.1: List of biofilm forming bacteria in biofilms-associated infections. Figure is adapted from [24] with permission.

P.aeruginosa is also highly capable for causing device-related infections including ventilator-associated pneumonia, central line-associated bloodstream infections, urinary catheter-related infections, and surgical/transplantation infections. [25-27] According to the International Nosocomial Infection Control Consortium, *P.aeruginosa* nosocomial infections have become a worldwide healthcare issue. [28] *P.aeruginosa* has also been reported to create the highest burden of healthcare-acquired infections in European intensive care units. [29] In the US, there are approximately 51,000 cases of healthcare-associated *P.aeruginosa* infections each year. [30] *P.aeruginosa* is highly prevalent in healthcare settings because it is usually found from patients under medical care. It has high survivability on disinfectant-treated abiotic and biotic surfaces while remaining transmissible from patient-to-patient. [31] *P.aeruginosa* infections are also becoming harder to treat because the bacterium is highly resistant to many classes of antibiotics. [30,32-33] There are limited therapeutic options for *P.aeruginosa* biofilms infections due to its diverse molecular resistance mechanisms, resulting in the increasing prevalence of multi-drug resistant cases. [33-35]

1.2 Antimicrobial resistance of biofilms

Biofilms are known to be highly resistant to antimicrobial treatment as compared to planktonic cells. [36-40] Extensive studies have been conducted to elucidate the resistance mechanisms of the biofilms. Some studies showed that biofilm matrix could function as a physical or chemical diffusion barrier to antimicrobial penetration into the biofilms. [41- 43] Other mechanisms include (i) the slow growth of biofilm due to a lack of nutrients in the environment; (ii) activation of general stress responses to cope with

the antimicrobial stress and (iii) the emergence of biofilm-specific phenotypes that reduce the effectiveness of the antimicrobial treatment. [6,10] These mechanisms will be further elaborated on the subsequent sub-sections.

1.2.1 Biofilm matrix as a barrier to antimicrobial penetration

Biofilm matrix is made up of eDNA, exopolysaccharides and other glycoproteins. The matrix has been postulated to prevent the diffusion of the antimicrobials into different parts of the biofilm. Roques and co. demonstrated that increased size of the hydrophobic side chains of the quaternary ammonium compounds within EPS matrix of *S.aureus* , reduced antibiotic susceptibility. When they removed the matrix, the antibiotic susceptibility was restored to the same level as that of the planktonic bacteria. [44] Dispersion and disaggregation of the biofilms also increased the susceptibility of the biofilms to antibiotics treatment. [6] These observations suggested that EPS matrix might confer protection to the biofilm by providing a physical protection that restricted the diffusion of the antimicrobials. This observation was further validated by another research group, which measured the amount of piperacillin penetrating through a dialysis membrane that had *P.aeruginosa* biofilms growing on one side of the membrane. The results showed that the biofilms prevented and restricted the diffusion of the antibiotics. [41] However, in another study, *Staphylococcus epidermidis* biofilms allowed the diffusion of vancomycin and rifampicin across the membrane, suggesting that the inhibition of the antibiotic penetration is not the sole mechanism for resistance. [45]

1.2.2 Slow growth and heterogeneity of biofilm

Nutritional limitations impede bacteria growth. Since most existing antibiotics are more effective against actively growing bacteria, the transition from exponential growth to lagged growth usually increased antibiotic resistance. [46,47] Bacterial cells embedded within biofilms were observed to have slow or no growth because of the lack of nutrients within the biofilms matrix. [48, 49] Furthermore, bacteria with different metabolic rates were observed within a biofilms, indicating heterogeneity in different part of the biofilms (Figure 1.3). For example, Molin's group used fluorescent-tagged cells to differentiate specific metabolic activities within the biofilm. They discovered that bacteria cells exhibit gradually reduced metabolic activity from the top layer to the centre portion of the biofilm microcolonies. . [50]

Figure 1.3: Different metabolic activity within a biofilm microcolony.

Figure adapted from [6]

The observation also demonstrated that nutrient availability is not uniform within a biofilm, thus affecting the metabolic activity of the bacteria cells. Other studies further supported the observation that decreased metabolic activity in some biofilms accounted for their enhanced resistance to antibiotic treatment. [51, 52] Bacterial cells at the top

interface of the biofilms are usually rapidly killed, where the dormant bacteria embedded deeply within the substratum remained alive and unaffected by antibiotic treatment. These surviving cells are able to form a nucleus for regrowth and recolonisation. [6]

1.2.3 Activation of general stress response

A recent study suggested that rather than nutritional limitations, slow growth within a biofilm can be attributed to the activation of a general stress response. [53] It is known that stress responses can induce physiological changes to the bacterial cells, protecting them from environmental stresses or chemical agents. [54] One of the central regulators of this stress response is the alternate σ factor, RpoS, which is induced under conditions of high cellular density. [55] RT-PCR result showed that RpoS mRNA is present in the sputum of CF patients infected by *P.aeruginosa* biofilms. [56] Another alternate σ factor, AlgT, has been reported to act in conjunction with RpoS to regulate the stress response. [53,56] Mutant variants of both alternate σ factors were found to be highly susceptible to hydrogen peroxide treatment when they were grown on alginate gel beads. [57] However, it was observed that as these variants formed thick biofilms, they became resistant to oxidative biocides, at similar level to the wild-type cells. This suggested that other factors are involved in the resistance mechanism as well. [10]

1.2.4 Induction of biofilm-specific phenotype

Studies have begun to reveal that a biofilm-specific phenotype is induced within the sub-population of the biofilms. This phenotype constitutively expresses an active resistance mechanism to reduce the effectiveness of antimicrobial agents. [58-60] An example of such resistance mechanisms is the expression of multidrug-efflux pumps. One study

suggested that one of these efflux pumps is crucial in conferring resistance to ofloxacin. [61] Biofilms lacking the pump were found to be more susceptible to ofloxacin as compared to biofilms that overexpressed the pump; there was no difference in susceptibility to ciprofloxacin. [61]

Another plausible resistance mechanism is the alteration of cell membrane structural integrity to restrict antimicrobial penetration. Such changes to the membrane-protein composition could decrease the permeability of the cells to antimicrobial agents. [40] For example, mutation of, *OmpB*, a regulator gene that controls the expression of outer membrane porin proteins (*OmpF*, *OmpC* and *OmpF*), decreases the susceptibility of *E.coli* to β -lactam antibiotic. [62] Furthermore, expression of *OmpC* and other osmotically regulated genes were found to be upregulated in biofilms in comparison with planktonic cell. [63] All these evidences suggested altered environmental conditions within a biofilm could lead to the development of biofilm-specific phenotypes that help to protect the bacteria from bactericidal activity of the antimicrobial agents.

1.3 Combination antimicrobial therapy to overcome biofilm resistance

Combination antimicrobial therapy is one of the conventional and effective approaches to overcome antimicrobial resistance and eradicate biofilm infection. [64] With the lack of novel antibiotics being introduced to the market in the recent years, combination therapy would be the obvious option to expand current antimicrobial treatment and their therapeutic potential. Combination therapy takes advantage of combining the different mechanisms of action and/or toxicity profiles of the antimicrobial agents to eradicate the

infection. This approach ensures that there (i) is at least one agent that is effective against the pathogen, (ii) is potential for synergistic activity and (iii) reduces the possibility of the developing resistance during treatment. [65]

Several studies had demonstrated the combination of colistin with other classes of antibiotic achieved improved therapeutic outcomes against *P.aeruginosa* biofilms as compared to colistin monotherapy. For example, colistin-tobramycin combination therapy is more effective than antibiotics monotherapy in eradicating *in vitro* *P.aeruginosa* biofilms. The combination also significantly reduced the bacteria count in rat lung infection models and in CF patients. [66] A similar result was also observed when a low dosage of colistin-tobramycin combination was used to treat *P.aeruginosa* PA14 biofilms in a murine tumor model. [67] Clinically relevant concentrations of doripenem also enhanced colistin-killing effects against biofilms in both carbapenem susceptible and resistance strains, meanwhile minimising the development of colistin resistance. [68]

Given the benefits of combinatorial antimicrobial therapy in the current stagnant state of antimicrobial innovation, it is imperative to constantly search for potential antibiotics and test new antimicrobial combinations to effectively eradicate and control biofilm-associated infections.

1.3.2 Other strategies for controlling biofilms

Besides combinatorial antimicrobial therapy, many other clinical methods for controlling biofilms had also been extensively researched. For example, the use of hypochlorous acid-based wound cleanser were shown to be more effective in disrupting and neutralizing methicillin-resistant *S.aureus* (MRSA) and *P.aeruginosa in vitro* biofilms with lower cytotoxicity than 0.05% chlorhexidine wound solution. [69] Similarly, high concentration of sodium hypochlorite was found to be the most active disinfectant against *P.aeruginosa* biofilms with 83.6% reduction of the biofilm biomass and 99.7% eradication of the live microbial cells. [70] Another common method to limit biofilm growth is wound debridement. However, commonly used topical antimicrobial wound solutions when applied for clinically relevant durations, were demonstrated to be ineffective against microbial biofilms *in vivo*. [71] A recent study also investigated the use of nitric oxide (NO) as an antibiofilm agent on soft contact lenses, and demonstrated effective dose-dependent inhibition of *S.aureus* and *P.aeruginosa* biofilms formation. [72] Finally, a new innovative method using aptamer-targeted nanotubes (aptamer-ciprofloxacin-single-walled carbon nanotubes) as an antibiofilm agent was demonstrated to be highly effective in eradicating *in vitro P.aeruginosa* biofilms. [73]

1.4 Knowledge Gap

According to the National Institute of Health (NIH) biofilms are associated with 80% of all microbial infections. [74] They are able to cause chronic infections because of their resistance to innate and adaptive immunity, as well as antibiotics. [36] Given the lack of new antibiotics to the market, there is an unmet clinical need for biofilm treatment. Even

though there was tremendous effort invested in exploring various anti-biofilm strategies such as quorum sensing inhibitor, interference in cyclic-di-GMP signaling, biofilm dispersal agents or weakening of the biofilm matrix, no biofilm-specific clinical treatment had been developed. [75] As a result, combination of antimicrobial therapy remains the most effective clinical option.

LpxC inhibitors are a new class of antimicrobial that possess potent and broad-spectrum activity. [76] LpxC inhibitors are currently undergoing extensive research by the pharmaceutical companies. [77] There is a huge development potential for LpxC inhibitors to meet future clinical need. However, the potency of these inhibitors was determined against planktonic bacteria but not biofilms. The efficacy and potency of these antimicrobials might not be applicable to the treatment of biofilms. Given that bacterial infections are usually associated with biofilms, it is only logical to study the effectiveness and mechanism of action of LpxC inhibitors against biofilms.

Currently, there is no *in vitro* or *in vivo* experimental data on the activity of LpxC inhibitors against biofilms. There is a knowledge gap concerning the effects of LpxC inhibitors against PAO1 biofilms as well as the stress response mechanisms of the bacterium when treated with LpxC inhibitors. Also, the combination potential of LpxC inhibitors with other classes of antimicrobials has yet been evaluated.

1.5 Hypothesis and objectives

We have selected one of the LpxC inhibitors, CHIR-090, to test in our study. It is a commercially available compound, which displays high potency against *P. aeruginosa* strains. Furthermore, many studies had used CHIR-090 as the reference compound, against which other CHIR-090-derived LpxC inhibitors are evaluated. [78-81] The chosen pathogen of this study is *P. aeruginosa* PAO1 because this species has been associated with biofilms infection in (CF) patients and was recently classified as one of the dangerous pathogens by the World Health Organization (WHO). [82]

In this study, we hypothesised that the disruption of cell membranes of Gram-negative cells due to the inhibition of the LpxC enzyme by CHIR-090 will increase the susceptibility of the biofilms to other antimicrobials, creating a synergistic potential when used in combination.

The first objective of the project is to examine the stress responses and possible resistance mechanisms of planktonic *P. aeruginosa* PAO1 to sub-inhibitory concentration of CHIR-090 using transcriptomic analyses and evolution studies. The overview of the transcriptomic profile and evolution study data would provide us with insights into the response of *P. aeruginosa* PAO1 to CHIR-090 upon treatment. This information can be useful in the elucidation of probable synergistic mechanisms of CHIR-090 when used with other antimicrobials.

The second objective is to evaluate the *in vitro* and *in vivo* effect of CHIR-090 alone, and in combination with colistin against PAO1 biofilms. Colistin is usually the last line of

antibiotics treatment against multidrug-resistant bacteria infections. Its toxicity has also limited the extensive use of colistin. [83] Many previous studies also focused on using colistin in combinational therapies, which showed increased efficacy in the killing of the biofilms. [66-68] Thus, it will be worthwhile to explore the possibility of expanding the current repertoire of colistin combinations to include CHIR-090.

The last objective of this project is to evaluate the *in vitro* and *in vivo* anti-biofilm activities of CHIR-090 in combination with other classes of antimicrobial such as macrolides (azithromycin), beta-lactams (carbencillin), aminoglycosides (tobramycin) and fluoroquinolones (ciprofloxacin). Ciprofloxacin and tobramycin are common antibiotics used to treat *P.aeruginosa* biofilm infections. [84] Studies showed that using combinations such as ciprofloxacin-colistin and tobramycin-colistin would increase the killing of the biofilms. [85] Given that CHIR-090 share a similar mechanism of action with colistin, it would be reasonable to investigate whether similar synergistic effect will be observed in the CHIR-090-tobramycin and CHIR-090-ciprofloxacin combinations. Azithromycin is a macrolide that inhibits quorum sensing in *P.aeruginosa*. [86] Studies had proposed to combine azithromycin with other antimicrobials to increase the killing potency against biofilms due to their anti-biofilm activity and their inhibitory effects on quorum sensing. [69] So, we will also be examining the effect of CHIR-090 in combination with azithromycin against PAO1 biofilms.

Overall, these studies will (i) demonstrate the potency of LpxC inhibitor, CHIR-090 alone against PAO1 biofilms, (ii) demonstrate the anti-biofilm effects of CHIR-090 in

combination with colistin and other antimicrobials, and (iii) provide evidences on the possible resistance mechanisms against CHIR-090.

1.6 Literature reviews

Since *P. aeruginosa* is used as the model organism in this study, it would be important to understand the different antibiotic resistance mechanisms in both of its planktonic and biofilms state. More specifically, knowledge regarding to the response of *P.aeruginosa* to different classes of antimicrobials would help us clearly and comprehensively understand the resistance mechanisms specific to each class of antimicrobials.

Many studies have been conducted to evaluate the effects of different antibiotic combinations against *P.aeruginosa* biofilms. These studies demonstrated the different efficacy and therapeutic potential of each antibiotic combination. Lastly, understanding the mechanism of action of LpxC inhibitor, CHIR-090, will provide useful information on the potential combinations of the compound with other classes of antimicrobials.

Here, we will give an overview of the current literature on antibiotic resistance mechanisms of *P.aeruginosa* in both planktonic and biofilms forms, the studies of LpxC inhibitors, particularly CHIR-090, and the current antibiotic combinations that have been used against *P.aeruginosa* biofilms.

1.6.1 Antimicrobial resistance mechanism of planktonic *P.aeruginosa* and biofilms

The antimicrobial resistance mechanisms of *P.aeruginosa* have been extensively studied and are broadly classified into three categories - intrinsic, acquired and adaptive. [87]

Intrinsic resistance refers to the existing resistance mechanism that regulates the susceptibility of the wild type organisms to antimicrobials. One example of intrinsic resistance is the outer membrane of Gram-negative bacteria; the semi-permeable outer membrane is an asymmetric membrane consisting of an inner leaflet of phospholipids and an outer layer of lipopolysaccharides (LPS). [88] According to Hancock and Bell, the permeability of the outer membrane of *P. aeruginosa* is about 100-fold lower than *Escherichia coli*, *E. coli*. [89] This low permeability of *P. aeruginosa* outer membrane limits and slows down the penetration of hydrophilic antibiotics such as β -lactams and quinolones through the porin channel. [87] While the outer membrane may limit the antibiotic uptake by the bacterium, it is not the only resistance mechanism since the antibiotic concentration will eventually reach equilibrium across the membrane.

Other intrinsic resistance mechanisms that commonly found in the *P. aeruginosa* include multi-drug efflux systems (e.g constitutively expressed *MexAB-OprM* and *MeXY-OprM*) and inactivating enzymes (e.g chromosomal β -lactamase *AmpC*). These mechanisms take advantage of the reduced penetration rate of antibiotics to pump out the antibiotics and/or degrade them as they diffuse across the membrane. [90, 91]

In addition to the intrinsic resistance mechanisms, *P. aeruginosa* can become less susceptible to antibiotics through acquired resistance via chromosomal gene mutation or

acquisition of resistance genes through horizontal gene transfer. [92] One of the common acquired resistance mechanisms in *P. aeruginosa* involves mutations leading to the loss or reduced activity of outer membrane porin channel, *OprD*. The mutated porin channel increased the bacteria's resistance to imipenem, requiring an up-to-16 fold increase in antimicrobial concentrations to achieve the desired inhibitory effects. [93] It was postulated that the mutation might have occur either on the *OprD* gene itself, or the regulatory gene, *mexT*. [93] Additionally, mutation of *mexT* also resulted in the constitutive activation of *MexEF-OprN*, conferring resistance not only to imipenem but other antimicrobials that are substrates of *MexEF-OprN* as well. [94] On top of the chromosomally expressed *AmpC* β -lactamase, *P.aeruginosa* was found to be able to acquire a plasmid-encoded, extended spectrum β -lactamase (ESBL) that is capable of hydrolysing various types of β -lactam antimicrobials. [95]

Recently, adaptive resistance has been increasingly recognised as an important mechanism of resistance. [87] Contrary to the intrinsic and acquired resistance mechanisms, occurrence of adaptive resistance is dependent on the environmental cues that trigger different regulatory responses in the bacteria. Also, the susceptibility of the bacteria to antimicrobials will be restored to normal levels when the antimicrobial stress is removed. This phenomenon was first observed by Greenwood as he demonstrated that bacteria exposed to sub-inhibitory concentration of antibiotics developed transient resistance to that particular antibiotic over time, and in some other cases, the similar resistance is extended to the same or even different classes of agents. [96]

Adaptive resistance has shown to be regulated by gene mutations that alter antimicrobial susceptibility, contributing to the overall resistance of the bacteria. [97-99] In a study by Brazas and Hancock, sub-inhibitory concentrations of ciprofloxacin treatment affected the expression pattern of more than 900 genes in *P.aeruginosa*, including genes that conferred resistance to ciprofloxacin. [98,100] Recent studies have also identified other types of adaptive resistance that involved the role of complex regulators such as heat shock-mediated adaptive aminoglycoside resistance, DNA-stress mediated adaptive fluoroquinolone resistance and two-component regulators *PhoQ*, *PsrA* and *CbrA* all of which, are capable of reducing susceptibility to multiple antibiotics. [101,102] One of the most characterised adaptive resistance mechanisms in *P.aeruginosa* is the resistance to polymyxins and cationic antimicrobial peptides by limiting extracellular Mg^{2+} and Ca^{2+} cations. [103,104] It was showed that alterations in the permeability of the outer membrane could be induced through *PhoPQ* and *PmrAB* regulatory pathways by the surrounding concentrations of cations, leading to the activation of tolerance to polymyxins and other cationic antimicrobial peptides. [105] Tolerance was developed by the addition of aminoarabinose to the phosphate groups of lipid A of LPS, decreasing the negative charges of LPS. This disrupted the interaction between polymyxin and the outer membrane. [106] There are also recent findings indicating that sub-inhibitory concentrations of polymyxins and cationic antimicrobials induced adaptive resistance via LPS modification, reducing the self-promoted uptake ability of the polycations. [104] Adaptive resistance is the most significant contributor to the discrepancies observed between *in vitro* susceptibility testing for *P.aeruginosa* isolates and clinical outcomes of infected patients. [87]

Antibiotic resistance in biofilms is different from planktonic bacteria. In biofilms, the antibiotic resistance is more complex and is a result of intrinsic, acquired and adaptive mechanisms. As mentioned in the introduction, *P. aeruginosa* biofilm cells are more resistant to antimicrobial agents than the planktonic cells. This is due to a range of factors such as adaptive gene expression changes influencing antibiotic susceptibility, low metabolic rate of the cells embedded within the biofilms and the higher concentration of antimicrobial degrading enzyme within the extracellular matrix. [10] Studies have also observed increased accumulation of acquired mutations that led to the development of antimicrobial resistance in both laboratory samples and heterogeneous population of *P. aeruginosa* in chronic lung infections. [107-110] Another unique phenomenon with *P.aeruginosa* biofilms is the presence of “persister” cells. These persisters were found to develop more effectively in the presence of the antibiotics and are considered as a major contributor to the resilience of biofilms-associated infections. [111-114] The reason for the development of such persister cells remains to be fully understood. However, there are evidences suggesting that the phenomenon might be caused by genetic changes induced within the bacterial cells after being exposed to antibiotic. [111, 113] The persister cells could also be an adaptive state induced by the stringent response in the biofilm. [115, 116] In the absence of antibiotics, these persister cells drive the re-development of the biofilms. [87] Further experiments need to be conducted to better understand the molecular mechanism behind the complexity of antibiotic resistance.

1.6.2 Stress response of *P. aeruginosa* to different classes of antibiotics

P.aeruginosa infections are hard to treat due to its various resistance (intrinsic, acquired and adaptive) mechanisms. [87] Inappropriate antimicrobial treatment readily selects for multidrug-resistant subpopulations of *P. aeruginosa*. Given that the bacteria can be exposed to a range of antibiotic concentrations during treatment, understanding the stress response of *P.aeruginosa* after exposing to sub-inhibitory concentration of antibiotics is crucial to optimise anti-pseudomonal chemotherapy. The primary biological responses of *P.aeruginosa* exposed to sub-inhibitory concentration of antibiotic are summarised in Table 1.2.

Sub-inhibitory concentrations of carbapenems (e.g.imipenem), fluoroquinolones and aminoglycosides had been shown to induce the formation of robust biofilms. [117-119] Ciprofloxacin had also been demonstrated to increase the frequency of mutation to β -lactams resistance. [120, 121] While macrolides are typically not effective against biofilms due to the outer membrane impermeability and the function of efflux pumps of *P. aeruginosa*, they were found to reduce virulence production factor. [86] Similarly, another class of antibiotic, tetracycline, that is relatively less effective against *P. aeruginosa*, induces biofilm formation and type-III secretion system, thus enhancing *in vivo* cytotoxicity of *P.aeruginosa*. [118] Sub-inhibitory concentrations of protein synthesis inhibitors such as aminoglycosides, tetracyclines and chloramphenicol induced the expression of *MexXY* multidrug efflux system. [122] Furthermore, induction of

MexCD-OprJ efflux system, which is dependent on *AlgU* stress response factor, was observed upon exposure to disinfectants such as chlorhexidine and benzalkonium. [123]

Antimicrobials	Biological responses	References
β -lactams	Induction of the AmpC β -lactamase (some β -lactams are inducers, but others are not)	Livermore (1995)
Carbapenems	Formation of thicker and more robust biofilms (induction of alginate biosynthesis)	Bagge et al. (2004)
Ceftazidime	Induction of mutagenesis and decreasing ciprofloxacin toxicity	Blazquez et al. (2006)
	Inhibition of quorum sensing	Skindersoe et al. (2008)
Fluoroquinolones	Induction of biofilm formation	Linares et al. (2006)
	Reduction in swimming and swarming	Linares et al. (2006)
	Induction of SOS response	Brazas and Hancock (2005)
	Up-regulation of the bacteriophage-like pyocins	Brazas and Hancock (2005)
	Shift from canonical DNA replication enzymes to inducible polymerases	Cirz et al. (2006)
	Enhancement of mutation frequency to β -lactam resistance.	Wolter et al. (2007), Tanimoto et al. (2008)
Protein synthesis inhibitors	Induction of the MexXY efflux system	Morita et al. (2012b)
Aminoglycosides	Induction of heat shock genes	Kindrachuk et al. (2011)
	Induction of biofilm formation	Hoffman et al. (2005)
	Induction of swimming and swarming	Linares et al. (2006)
	Induction of the Lon protease	Marr et al. (2007)
Macrolides	Quorum sensing antagonistic activity (reduction in virulence factor)	Tateda et al. (2001)
	Induction of the T3SS	Nalca et al. (2006)
	Down-regulation of the MexAB-OprM pump	Sugimura et al. (2008)
Tetracycline	Induction of biofilm formation	Linares et al. (2006)
	Induction of the T3SS and cytotoxicity	Linares et al. (2006)
Chloramphenicol	Induction of the MexEF-OprN efflux pump	Fetar et al. (2011)
Polymyxins	Modification of LPS lipid A with 4-amino-L-arabinose	Fernandez et al. (2010)
	Up-regulation of the PQS biosynthetic genes	Cummins et al. (2009)
Chlorhexidine	Induction of the MexCD-OprJ efflux pump	Morita et al. (2003)
	Induction of the oprH-phoPO operon	Nde et al. (2009)

Table 1.2: Stress/Biological responses of *P. aeruginosa* exposed to different classes of antibiotics at sub-inhibitory concentration. Figure is adapted from [124] with permission

Overall, clinically useful anti-pseudomonal antimicrobials such as carbapenems, fluoroquinolones and aminoglycosides are effective in killing *P. aeruginosa* at the inhibitory concentrations; these agents induced biofilms formation upon exposure to sub-inhibitory concentrations. This would mean that optimisation of anti-pseudomonal

antimicrobial therapy is crucial to reduce the possibility of developing multidrug resistance.

1.6.3 Current antibiotic combination treatments against *P. aeruginosa* biofilms

Combinations of antibiotics are commonly used in the clinical setting since they help enhance the effectiveness of each drug at lower concentration, and reduce the likelihood of resistance development. The individual drugs are often structurally distinctive classes of antibiotics that target different pathways of bacterial propagation (metabolic, signaling and biological pathways); they are usually selected for their synergistic activity. While many antibiotic combinations have been tested against planktonic *P. aeruginosa*, few have been tested against Gram-negative bacteria biofilms. More extensive studies will be required to optimise combinations with existing antibiotics against *P. aeruginosa* biofilms.

Combination antimicrobial therapy remains one of the most effective therapeutic strategies to overcome *P. aeruginosa* biofilms infections. For example, antibiotic combinations have shown to be effective in combating *P. aeruginosa* pulmonary biofilm infections. [66, 125, 126] Polymyxins antibiotics, such as colistin, are now being used as a last resort antibiotic to eradicate *P. aeruginosa* biofilms. The combination of colistin and tobramycin has been demonstrated to be highly effective against *P. aeruginosa* biofilm infections in clinical trials. [66, 127] Another promising combination is that of tobramycin and clarithromycin. With each individual antimicrobial agent having

moderate activity against biofilms and planktonic cells, the combination of these two antibiotics have shown to be synergistic and is significantly more effective in clearing biofilm infections. [125] On the other hand, the combination of tobramycin and azithromycin has been shown reduced effectiveness towards *in vitro* biofilms as compared to the monotherapy of each antibiotic while the combination of tobramycin and aztreonam did not show any additional efficacy over tobramycin monotherapy. [126, 127]

Targeting different sub-populations within the biofilms is one of the strategies to achieve synergy in eradicating biofilms. For example, ciprofloxacin and tetracycline are known to target the metabolically active subpopulation of the biofilms, while colistin targets the metabolically dormant subpopulation. The combination of colistin and ciprofloxacin achieves synergy by targeting two distinct populations of the biofilms. [85]

Research efforts have been ongoing to create more potent conventional treatment against biofilms by modifying existing antibiotics. For example, the new antipseudomonal cephalosporin CXA-101 had demonstrated effectiveness against multi-drug resistant strains of *P. aeruginosa* in biofilm infections and respiratory infections. [129-131]

However, the biggest challenge in the eradication of biofilms remains with its resilient nature and the ever-adapting resistance mechanism. This necessitates further development of more specific anti-biofilm therapeutic options, as well, as further

evaluation of existing antibiotics for effective combinations that can eradicate *P.aeruginosa* biofilms.

1.6.4 Development of LpxC Inhibitors and CHIR-090 derivatives

In the recent years, an essential zinc-dependent metalloamidase, UDP-3-O(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase, LpxC has been identified to be a potential antimicrobial target. [71] LpxC is one of the nine unique enzymes involved in the biosynthesis of Lipid A (Figure 1.4).

Figure 1.4: Overview of Kdo₂-Lipid A biosynthesis pathway. Figure is adapted from [132] with permission.

Lipid A is made up of glucosamine-phospholipid, which forms the outer monolayer of Gram-negative bacteria (Figure 1.5). It is the membrane anchor for lipopolysaccharide (LPS) and plays a protective role against antibiotics and detergents. [133]

Figure 1.5: Schematic structure of Gram-negative bacteria cell envelope. Figure is adapted from [132] with permission

Bacterial cells with mutation in lipid A are non-viable while reduced level of lipid A biosynthesis increases its susceptibility to wide range of antibiotics. [134] Furthermore, lipid A is also a potent endotoxin that is capable of eliciting septic shock with high fatality rate. [135, 136] Hence, small molecules that target biosynthesis of Lipid A can

cure Gram-negative infection and may also increase susceptibility of the bacteria to other classes of antibiotics. In addition, by targeting lipid A endotoxin, they would also prevent the patients from suffering from septic shock. [71]

The first committed step of lipid A biosynthesis is the deacylation reaction by LpxC enzyme. While the first six enzymes of the reaction are considered to be potential drug targets, LpxC stands out among the rest because of its regulatory role in the biosynthesis of lipid A. [137, 138] Furthermore, this enzyme is conserved among Gram-negative bacteria and shares no homology with the eukaryotic cells. [71] These features of the enzyme permit the development of inhibitors with high specificity and limited side effect.

Several competitive LpxC inhibitors were discovered to have binding affinity to the LpxC enzymes, but not all have antibiotic activity. The first LpxC inhibitor, **L-161, 240**, was discovered by Merck Research Laboratories (Figure 1.6). This competitive inhibitor has a dissociation constant (K_i) of 50 nM for EcLpxC. [139] **L-161, 240** was shown to be more effective inhibiting *E.coli* growth as compared to rifampicin and erythromycin and found to protect mice against septicaemia. [139] However, the compound was not effective against *P.aeruginosa* and had narrow antibiotics spectrum. [139] To develop a broad-spectrum LpxC inhibitor, Jackman's group designed a series of chemical structures mimicking the hexose and acyl chain moieties. [140] Out of this series, **TU-514** was found to inhibit the broadest range of LpxC activities such as AaLpxC (*Aquifex aeolicus*), EcLpxC (*E.coli*) and PaLpxC (*P.aeruginosa*) and was the one of the most

potent LpxC inhibitors (Figure 1.6). [140] However, this compound has no antibiotic activity due to the long hydrophobic acyl chain preventing cell membrane penetration by the compound. [141]

Figure 1.6: Structures of the early LpxC inhibitors. Figure is adapted from [71] with permission

There was a major progress in the search for LpxC inhibitors when two novel hydroxamate-based, branched sulfonamide compounds were discovered. [140] **BB-78484** and **BB-78485** have two hydrophobic moieties around a hydromxamate core (Figure 1.6). The dissociation constants of **BB-78484** and **BB-78485** were estimated to be 50 nM and 20 nM for EcLpxC respectively [140] These two compounds were able to inhibit the growth of a range of Gram-negative bacteria but were not active against *P.aeruginosa*. [140] Lastly, a novel compound called *N*-aroyl-L-threonine hydroxamate, **CHIR-090** was unveiled by Andersen's research group and was characterized by Mcleren group. [141, 143] **CHIR-090** is patented to Chiron and the University of Washington (International patent WO 2004/062601 A2) and was the most potent LpxC-targeting compound among the inhibitors described previously. [143] Unlike the previously described LpxC inhibitors, which bind to and instantaneously dissociate from LpxC enzyme, CHIR-090 is a "two-steps, slow and tight binding LpxC inhibitor" to AaLpxC with a K_i of 1 nM and followed by an irreversible enzyme/inhibitor isomerisation. [141] Results obtained from disc diffusion showed that the compound is able to inhibit the growth of *E. coli* and *P. aeruginosa* with efficacy similar to ciprofloxacin and tobramycin (Figure 1.7). However, due to its narrow therapeutic spectrum and rapid development of pathogen resistance, CHIR-090 was not developed for commercial use. [144] Instead, the compound was investigated for its structural activity relationship (SAR) between CHIR-090 and LpxC enzyme. [141] The SAR study

provided critical information for future modifications of CHIR-090 to improve its potency and expand the therapeutic spectrum.

B.

Figure 1.7: The antibiotic activity of the L-161,240 and CHIR-090 on *E.coli* W3110 bacterial lawn . Figure is adapted from [71] with permission.

In the recent years, there is continuous effort to improve the design and development for more potent and clinically relevant LpxC inhibitors. Zhou's research group reported the biochemical and structural characterisation of modified derivatives based on the linear diacetylene scaffold of CHIR-090. [145] These compounds exhibited excellent potency in reducing the resistance of RILpxC (*R.leguminosarum*) as compared to the original CHIR-090 (Figure 1.7). Out of these compounds, **LPC-009** showed increased potency of up to 64 folds over CHIR-090 against many clinically relevant Gram-negative pathogens

such as *E.coli*, *P.aeruginosa*, *Klebsiella pneumonia*, *Vibrio Cholera*, and *Burkholderia species*. [145] Another group presented a novel series of alkyl hydroxamic acid LpxC inhibitors that exemplified by compound **1a** (Figure 1.8). These compounds exhibited potent and selective enzyme inhibitor activity, broad-spectrum activity against Gram-negative bacteria like *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *Acinetobacter baumannii*, and *in vivo* efficacy in a murine infection model. [146] Two of the LpxC inhibitors from this series were tested against clinically relevant strains of *P. aeruginosa* and demonstrated better efficacy compared to all the current antimicrobials used in the study except for polymyxin B, which is the last option for the treatment of multidrug-resistant *P.aeruginosa* infections. [147] The key feature of this series of inhibitors is the methylsulfone-containing headgroup, which forms a hydrogen-bonding interaction with the active site residue Lys238. [147] The same group also introduced a pyridine-substituted compound, **LpxC-4**, shown to be a potent, broad-spectrum inhibitor that has very low frequency of the development of spontaneous resistance (Figure 1.7). The compound also demonstrated *in vivo* potency in both acute septicaemia and neutropenic thigh infection models. [148]

Figure 1.8: The chemical structure of activity of CHIR-090 derivatives. Figures are adapted from [145-148] with permission.

1.6.5 LpxC inhibitor – N-aryl-L-threonine hydroxamate, CHIR-090

CHIR-090 is the most potent LpxC-inhibitor among the early-discovered LpxC inhibitors. University of Washington (UW) and Chiron discovered the compound after screening 1200 synthesized compounds against *P.aeruginosa* LpxC in an *in vitro* enzymatic assay. [149]

Figure 1.9: Structure of *A. aeolicus* LpxC-CHIR-090 complex.

Figure is adapted from [145] with permission.

The compound is a two-step slow, tight binding inhibitor of the EcLpxC (*Escherichia coli*) and AaLpxC (*Aquifex aeolicus*) with K_i of 4.0 nM and 1.0-1.7 nM respectively. [141,150] While CHIR-090 formed an irreversible enzyme/inhibitor isomerization with AaLpxC, the compound inhibited EcLpxC in a fully reversible manner with K_i of 5 nM and dissociation constant, K_i^* of 0.5 nM after isomerization. [141,150]

Binding specificity of CHIR-090 to AaLpxC enzyme has also been extensively characterized. LpxC enzyme is a novel “ β - α - α - β ” sandwich fold where the four alpha helices located internally are sandwiched between two beta sheets. (Figure 1.9) Two domains of the molecule have similar fold with each containing one

five-stranded β -sheet and two α -fold helices. [151] However, the β -sheet of Domain I is severely distorted while the sheet of Domain II is relatively flat [151]. Each of the domains also contained a unique insert with Insert I of Domain I make up of a small “ β -sheet” while Insert II of Domain II consisting of a β - α - β fold. [145]

Structural data showed that CHIR-090 interacted with the LpxC enzyme at three distinct regions (Figure 1.10). The first region is the coordination of the hydroxamate moiety of CHIR-090 with catalytic zinc ion [145]. The binding of CHIR-090 is further strengthened through interactions with conserved hydrophilic and hydrophobic residues at the active site (Figure 1.10). The second region involved the insertion of CHIR-090 into the LpxC hydrophobic passage with the end of the molecule protruding from the opposite end of the passage. Extensive nuclear overhauser effect, NOEs are observed between CHIR-090 with residues (e.g. I18, I186, I189, G198, T203, and V205) within the passage. [145] The last region is the fitting of the morpholine ring of CHIR-090 to the shallow hydrophobic pocket at an approximately 110° angle relative to the biphenyl acetylene moiety. (Figure 1.10) It was hypothesized that the presence of strong NOEs between morpholine ring and methyl groups (I186, L200, V205 and Y212) along the passage bounded morpholine ring by hydrophobic interaction. [145]

Figure 1.10. Binding interface between LpxC with CHIR-090 (A) CHIR-090 occupies the LpxC hydrophobic passage with the hydroxamate moiety interacting with the zinc ion - Hydrophobic surfaces are coloured in orange. (B) Position of the CHIR-090 threonyl and hydroxamate groups in active site. Figures are adapted from [145] with permission.

The compound demonstrated strong antibiotic activity against *E.coli*, *P.aeruginosa* and was also active against all representative strains of *B.multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B.ambifaria*. [141,150,152]. However, CHIR-090 is less effective against *R.leguminosarum* LpxC with K_i of $0.34\mu\text{m}$, thus does not control the growth of *R.leguminosarum* or related bacteria in *Rhizobiaceae* family. [150] And currently, no combination antimicrobial therapy study has been conducted using CHIR-090 with other existing antimicrobials.

In order to maximize therapeutic potential, studies were conducted to investigate the potential mechanisms that may lead to the development of mutationally acquired resistance to CHIR-090. Various *P. aeruginosa* efflux pump mutants were tested to show

that CHIR-090 is a substrate for MexAB-OprM, MexCD-OprJ and MexEF-OprN. [153] Similarly, *in-vitro* overexpression of LpxC enzyme decreased the susceptibility of *P.aeruginosa* to CHIR-090 while amino acids substitution of the residue within the active site of LpxC increased the MIC of CHIR-090. [153] In the same study, they also identified a mutated fatty acid biosynthetic pathway gene *fabG*, which resulted in a slow growing mutant that played an important role in conferring resistance to CHIR-090.

In another study, isolated *E.coli* mutants with mutated *fabZ* and *thrS* gene were found to confer 200-folds more resistant to LpxC inhibitors (including CHIR-090) than the wild-type strain through a rebalancing cellular homeostasis mechanism. [154] They observed a decreased level of LpxC enzyme in *fabz* mutant bacterial cells without the presence of LpxC inhibitors, suggesting a tight regulation between biosynthesis of fatty acid and lipid A to maintain the membrane composition balance. [154] Additionally, mutation in *thrS* reduced protein biosynthesis, exerting a suppressive effect on inhibition of membrane biosynthesis. [154] Overall, both *P.aeruginosa* and *E.coli* are able to employ various resistance mechanisms to reduce the *in vitro* susceptibility to CHIR-090.

Chapter 2: Genome and transcriptome analysis of *Pseudomonas aeruginosa* PAO1 treated with sub-inhibitory concentration of LpxC inhibitor, CHIR-090

P.aeruginosa is a gram-negative, opportunistic pathogen with broad-host range. It is becoming a serious threat and also harder to treat because of limited and decreasing effective therapeutic options. [155, 156] This gram-negative pathogen is a common cause of burn-wound infections, urinary tract infections and chronic infection in cystitis fibrosis patients. [157]

This organism has intrinsic resistance to most of the currently available antibiotics and is capable to rapidly develop resistance to anti-pseudomonal agents. The unique combination of impermeability of the outer membrane and efflux pumps such as resistance-nodulation-cell division (RND) family has aided *P. aeruginosa* to resist to any antimicrobial or toxin attack. [158, 159] In particular, the comparatively less permeable outer membrane (OM) has enhanced the ability of the efflux pump to reduce the intracellular accumulation of the toxic molecules. [160] Besides being a permeable barrier to toxin molecules, OM plays an essential role in the viability and virulence of *P. aeruginosa*. [161, 162] Given that OM is essential for bacterial survival, OM synthesis is an attractive drug target for antimicrobial development. One of the important components of the outer leaflet of OM is lipopolysaccharide (LPS). The membrane anchor of LPS on the outer leaflet is lipid A and the synthesis pathway of lipid A has been the focus for developing a potential inhibitor. The role of lipid A is necessary and

structurally conserved among the Gram-negative bacteria. Among all the enzymes involved in the synthesis of lipid A, LpxC, which encodes for UDP-3-O-acyl-N-acetylglucosamine deacetylase, is involved in the first committed step in the synthesis of lipid A and thus makes it an ideal drug target. [71, 132]

The search for LpxC inhibitors had brought about promising candidates with potent antimicrobial activity. [71] One of these inhibitors, CHIR-090, was found to be the most potent LpxC inhibitor which demonstrated potency against *P. aeruginosa* with efficacy that is comparable to ciprofloxacin and tobramycin. [71, 141] Resistance mechanism of the bacteria limits the efficacy of new antimicrobial, hence understanding potential resistance mechanisms of the antimicrobial is important for optimization process. Previous studies attempting to look for possible *in vitro* resistance mechanisms in *P.aeruginosa* had identified that efflux pumps, overexpression of LpxC enzyme and alteration in fatty acid synthesis were various ways to decrease susceptibility to CHIR-090. [153, 154] However, in those studies, the development of resistance mechanisms over a long treatment period within the bacteria populations was not characterized. Also, there is currently no transcriptomic analysis of *P.aeruginosa* to give a better understanding of the stress responses when the bacteria are treated with CHIR-090.

Hence, in this chapter, we did a preliminary study aiming to identify other possible mechanisms that may lead to mutational acquired resistance to CHIR-090 through an evolution study. Furthermore, we also conducted a transcriptomics analysis to investigate the stress responses of *P. aeruginosa* upon treatment with CHIR-090. Data obtained

from this analysis can be useful to elucidate the potential resistance mechanism or synergy mechanism with other antimicrobials.

2.1 Materials and Methods

2.1.1 Bacterial strains and media

The lab strain, PAO1 and its CHIR-090-resistant derivative, 090-TJH, were tested in this study. 090-TJH was generated by continuous exposure to sub-inhibitory concentration of 0.5 µg/ml of CHIR-090 for 21 days. Muller-Hinton Broth (MNB, Difco™, Fisher Scientific, SAS, Illkirch, France) was used for evolution assay (bacteria exposed to sub-inhibitory concentration of antimicrobial for 21 days) and RNA sequencing transcriptomic analysis.

2.1.2 Preparation for RNA-sequencing samples and transcriptomic analysis

For transcriptomic analysis, triplicates of PAO1 samples treated with either DMSO or 0.5 µg/ml of CHIR-090 were cultured till early stationary phase. 0.8mL of each culture were harvested and mixed immediately with 2 to 4 volumes of RNeasy Protect® Bacteria reagent (Qiagen). The samples were then incubated for 5 to 20 minutes at room temperature. Subsequently, after incubation, the samples were centrifuged at 10,000 x g for 5 minutes at 4°C to isolate the bacteria pellet and immediately kept until use at -80°C.

The total bacterial RNA was purified with miRNeasy Mini kit (Qiagen). RNase-free DNase set (Qiagen) was used to remove DNA contaminant through an on-column DNase Digestion. Total RNA and DNA quality and concentration were measured by Agilent

2100 Bioanalyzer (Agilent Technologies) and Qubit® 2.0 fluorometer (Invitrogen). Gene expression analysis of the samples was carried out using Illumina RNA sequencing (RNA-seq). The libraries were prepared using Illumina TruSeq RNA Sample Prep Kit and were sequenced using the Illumina HiSeq 2500 platform with a paired-end protocol and read length of 100 nucleotides. The genes were annotated using either GO annotation or RAST annotation. [163–165]

RNA-sequenced data were analyzed by Dr. Gurjeet Singh using the protocol as described in Chua et al 2014. [166] Briefly, the sequence reads were mapped onto the template genome using “RNA-Seq and expression analysis” application in the CLC genomics Workbench 9.0 (CLC Bio, Aarhus, Denmark). Statistical analysis of the transcript count table was conducted by DESeq2 package of R/Bioconductor. [167, 168] Transcript counts were normalized to the effective library size and differentially expressed genes were identified with a negative binomial test. Differentially expressed transcripts were determined by stringent requirement of a fold change larger than 2 and an adjusted p-value smaller than 0.05. Heatmap and Principle component analysis (PCA) was drawn and plotted using heatmap.2 package of R/Bioconductor and online web tool <http://biit.cs.ut.ee/clustvis/>. [169]

2.1.3 Sequencing, assembly and annotation of 090-TJH resistant population

Genomic DNA from three populations subjected to 0.5 µg/ml of CHIR-090 was purified using QIAmp DNA Mini Kit (Qiagen) and sequenced on an Illumina HiSeq 2500 platform. The sequencing data from each of the population were to identify single

nucleotide polymorphism (SNP) by mapping to the PAO1 genome (NC_002516.2) using CLC Genomic Workbench 10.0 (CLC bio, Qiagen). Genomic sequencing was performed by Dr. Ding Yichen.

2.2 Results

2.2.1 Statistical analysis of transcriptomic data of PAO1 treated with CHIR-090 or DMSO

The integrity of RNA-seq data was validated with the quality-control test such as Principle Component Analysis (PCA) before analyzing differentially expressed genes. Each dot on the PCA plot represents the total transcript of each sample. (Figure 2.1) According to PCA analysis, principle component 1 accounted for 53% of while principle component 2 accounted for 21.6% of the total variance. (Figure 2.1) From the PCA plot, there was a distinction between each sample replicates along PC1. Both the control and the treated group showed greater variation along PC2. The small difference in proximity within the replicates of the same group indicated the low variation in the transcriptomic profiles of the triplicate in the individual treatment group. The large distinction between the locations of replicates of the two treatment groups indicated the different transcriptomic expression profiles of the two treatment groups. The overall differential transcriptiomic expression of the two samples was visualized using heatmap plotted with Z-scores. (Figure 2.2)

Figure 2.1: PCA plot of genes in planktonic *P.aeruginosa* treated with DMSO (con) or subinhibitory of CHIR-090 (comp). Fold change ≥ 2 , p-value ≤ 0.05 , n =3. Credit to Dr. Gurjeet Singh for PCA plot analysis

Figure 2.2: Heatmap of gene expression in planktonic *P.aeruginosa* treated with DMSO (con) or subinhibitory of CHIR-090 (comp). Similar patterns were observed among the replicates while distinct difference in expression profile of the two different treatment

groups. Fold change ≥ 2 , p-value ≤ 0.05 , n =3. Red colour: Downregulated genes; Blue colour: upregulated genes. Credit to Dr. Gurjeet Singh for heatmap analysis.

2.2.2 Genes up-regulated in PAO1 treated with CHIR-090

Parallel comparison was conducted between the two treatment groups to determine the expression of the genes. The up-regulated genes of the CHIR-090 are listed in Table 7.1.1. With the selection criteria of fold change ≥ 2 and p-value ≤ 0.05 , there were 106 genes affected after the treatment with CHIR-090. Out of 106 genes, 76 genes were upregulated (Table 7.1.1). Among all these upregulated genes, genes that are involved in fatty acid biosynthesis and efflux pump expression were most upregulated. Genes involved in fatty acid biosynthesis like *faoA* and *faoB* were upregulated for 7.3 folds and 10 folds and the transcriptional regulator, *psrA* involved in regulating the transcription of *faoA* and *faoB* was upregulated for 5.3 folds. The next group of the most up-regulated transcribed genes was the resistance-nodulation-cell division (RND) multidrug efflux pump. Genes such as *mexC*, *mexD* and *OprJ* forming the *mexCD-OprJ* efflux pump were upregulated by 4.5 folds to 6.1 folds. Interestingly, genes of two component regulatory systems, *phoP* and *phoQ*, in *P. aeruginosa* were slightly upregulated by 2 folds and 2.2 folds respectively. Similarly, *oprH* gene that is part of the *phoP-phoQ-oprH* operon encoding for outer membrane protein was upregulated by 3.7 folds. Gene involved in divalent cation uptake like *mgtA* was upregulated by 3.2 folds. Genes such as *glpD*, *glpF*, *glpK* and *glpT* involved in glycerol metabolism were upregulated by 2 folds to 3.73 folds. *NarkI* gene involved in transportation of nitrate was upregulated by 3.5 folds. Genes

encoding electron transfer flavoprotein, *etfA* and *etfB*, were also upregulated by 2.4 folds and 2.1 folds, respectively.

2.2.3 Genes down-regulated in PAO1 treated with CHIR-090

The down-regulated genes of the CHIR-090 are listed in Table 7.1.1. With the selection criteria of fold change ≥ -2 and p-value ≤ 0.05 , there were 106 genes affected after the treatment with CHIR-090. Based on the transcriptomic analysis, 30 genes were downregulated (Table 7.1.2). The majority of the genes downregulated belongs to *cys* gene clusters (*cysA*, *cysD*, *cysH*, *cysT* and *cysW*) with a fold change from -2.1 to -3.7. These genes are associated with central metabolism that functions as a metabolic link between nutrient metabolizing and biosynthesis. Genes involved in amino acid metabolism like *gcdH* and *aspA* were downregulated by 2 folds. Gene along nucleotide metabolism like *cpdA* was downregulated by 2.5 folds. Interestingly, *phzA2* gene, encoding a set of proteins that catalyze the synthesis of phenazine-1-carboxylic acid, which is a precursor to produce various toxin phenazine derivatives, was downregulated by 2.4 folds.

2.2.4 Genomic analyses of CHIR-090 resistant strain, 090-TJH

To identify other possible resistance mechanisms that might arise during long period of CHIR-090 treatment, evolution assay was carried out by treating three populations of PAO1 with sub-inhibitory concentration of 0.5 μ g/ml of CHIR-090 for 21 days till their MIC reached 16 μ g/ml or 32 μ g/ml (MIC of CHIR-090 is 1 μ g/ml). The genome of these three populations was sequenced and only unique mutations that were identified are

reported in Table 2.1. The complete genomic mutation list of three populations can be found in the appendix 7.2. A total of six unique mutations that might associate with antimicrobial resistance were identified in the three populations. These mutated genes are categorised into three groups; efflux pump, cell wall/membrane/lipopolysaccharides synthesis and motility. However, not all three populations had the same mutations. *mexR* was the only gene that found to be mutated in all three populations. Mutated *murB* gene was observed in two of the populations while *pyrG*, *fabF₁*, *fliF* and *morA* were unique mutation observed in one population.

Gene name	Coding region change	Amino acid Change	Non-synonymous	Type of mutation	Function/Reference
Efflux Pump					
<i>mexR</i>	NP_249115.1 :c.209G>T	NP_249115.1 :p.Arg70Leu	Yes	SNV	multidrug resistance operon repressor MexR [170]
Cell wall/membrane/lipopolysaccharides synthesis					
<i>murB</i>	NP_251667.1 :c.728A>G	NP_251667.1 :p.Asn243Ser	Yes	SNV	UDP-N-acetylenolpyruvoylglucosamine reductase [171]
<i>pyrG</i>	NP_252327.1 :c.592C>A	NP_252327.1 :p.Arg198Ser	Yes	SNV	CTP synthetase [172]
<i>fabF₁</i>	NP_251655.1 :c.812C>T	NP_251655.1 :p.Thr271Ile	Yes	SNV	3-oxoacyl-ACP synthase [173]
Motility					
<i>fliF</i>	NP_249792.1 :c.147_148de ICG	NP_249792.1 :p.Ala50fs	Yes	Deletion	flagellar MS-ring protein [174]

<i>morA</i>	NP_253291.1 :c.2894G>A	NP_253291.1 :p.Ser965Asn	Yes	SNV	motility regulator [175]
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Table 2.1: List of genes mutated in the 090-TJH populations. Gene name, coding region change, amino acid change, non-synonymous, type of mutation and product were shown in table. Credit to Dr Ding Yichen for the genomic analysis

2.3 Discussion

Pseudomonas aeruginosa is a gram-negative pathogen that is capable of causing various clinical conditions and chronic infections to immunocompromised individuals. [155, 156] This bacterium is well known for its resistance to antibiotic treatment through its highly effective resistance-nodulation-cell division (RND) family efflux pump and its impermeable outer membrane (OM) that prevents the entry of the foreign toxins and antimicrobials. [161,162] In short, the OM enhanced the ability of the efflux pump to reduce the intracellular accumulation of the toxic molecules. More importantly, OM is vital to viability and virulence of the bacteria, making the OM an attractive drug target. The outer leaflet of the outer membrane is composed of LPS. LPS is made up of an anchor protein, lipid A, a nonrepeating core polysaccharide and a repeating O antigen polysaccharide chain that extend outward from the cell surface. [132]

In the recent years, the synthesis pathway of lipid A has attracted much attention as a possible drug target pathway. [161] LpxC, the first committed enzyme in the synthesis of lipid A, is identified to be an ideal target given that this enzyme is relatively conserved among other gram-negative bacteria. [132, 161] Among the LpxC inhibitors discovered, CHIR-090 was the most potent against *P. aeruginosa* with efficacy comparable to ciprofloxacin and tobramycin. [71] The therapeutic effectiveness of a new antimicrobial might be reduced by possible emergence of resistance mechanisms. Hence, it is critical to understand the possible resistance mechanisms in the early stage of development in order to optimize the development and maximize its use.

In this study, we aimed to study the stress response profile of PAO1 during CHIR-090 treatment. Using RNA transcriptomic analyses, expression of 106 genes was altered significantly by sub-inhibitory concentration of CHIR-090. Expression of 76 genes was upregulated while 30 genes were downregulated. Among the upregulated genes, three gene clusters, which were highly expressed, might contribute to the stress responses during the treatment.

The first upregulated gene cluster was the *faoAB* operon involved in fatty acid beta-oxidation with its *psrA* transcript regulator that binds to the promoter region of *faoAB*. [176, 177] A previous study has shown that *faoA* mutant of *P.aeruginosa* has increased susceptibility to aminoglycosides while complementation of the mutant with the wild type gene restored its resistance to the level similar to the wild type. [178] Furthermore, *faoAB* transposon mutants also displayed increased susceptibility towards tobramycin. [179] This suggests that *faoAB* has the ability to confer resistance against CHIR-090. Given that *faoAB* operon was the most upregulated (~7 to 10 folds) gene cluster, we suspected that *faoAB* genes might be responsible for one of the primary stress responses during CHIR-090 treatment. While further experiments will be required to validate this observation, we hypothesized that *faoAB* mediated FA oxidation gene might have modulated the fatty acid composition of the bacteria by replacing short and unsaturated fatty acids with longer and saturated fatty acids in response to membrane perturbants like β -lactam antimicrobial in order to stabilize the membrane structures and limit the perturbation of the compound. [178]

The second cluster of up-regulated genes was *mexC*, *mexD* and *OprJ* that encodes for resistance-nodulation-cell division (RND) family efflux pump. [180] *MexCD-OprJ* is a multidrug efflux pump commonly expressed in *P. aeruginosa*. *mexC* encodes for membrane fusion protein; *mexD* encodes for a membrane transporter and *OprJ* encodes for outer membrane channel. *MexCD-OprJ* efflux pump in *P. aeruginosa* was previously known to display resistance to antimicrobials such as the quinolones, chloramphenicol, tetracyclines and macrolides. [181-183] The general role of the efflux pump is to export molecules such as antimicrobials out of the bacterium through a process driven by the inner membrane potential gradient, limiting the intracellular accumulation of foreign molecules. [184] From the analysis, the upregulation of the expression of *MexCD-OprJ* efflux pump demonstrated that efflux pump might be another stress response activated during CHIR-090 treatment. This observation was consistent with an *in vitro* study, which showed CHIR-090 is a substrate of *MexCD-OprJ* efflux pump. [153] Furthermore, it was proposed that during envelope stress (given that CHIR-090 targets the membrane), *AlgU*-dependent induction of *P.aeruginosa mexCD-oprJ* efflux pump might be activated to upregulate the expression of *mexCD-oprJ* to export the damaged or released fatty acids as part of the mechanism to restore its cell membrane stability. [185] Given that the transcriptomic analysis showed a ~2 folds increase in *AlgU* transcription, we postulated that this might be one of the possible resistance mechanisms.

The last cluster of genes that was upregulated was the two-component regulatory system and an outer membrane protein - *phoP*- *phoQ* and *oprH*. Previous study showed that *phoP-phoQ* regulated the expression of *oprH* and conferred polymyxin resistance in

response to the changes in Mg^{2+} concentrations in the environment. [103] Also, *phoP-phoQ* system is known to regulate another two component regulatory system, *pmrAB* system that mediates the biosynthesis of 4-amino-4-deoxy-L-arabinose (Lara4N). [186] Modification of LPS occurs when Lara4N is added to the phosphate groups of lipid A, thereby increasing the charge of lipid A, resulting in the reduction of affinity of positively charged polymyxins. [187] Based on the transcriptomic data, the most likely stress response induced might be due to the increased expression of *oprH* (~3.7 folds) and not the *pmrAB* pathway because there was no upregulation of the *pmrAB* expression. The probable mechanism of the stress response might be due to the interaction of *OprH* with LPS, increasing the stability of the outer membrane, and thus conferring the antibiotic resistance. [188]

Among 30 downregulated genes, a cluster of genes (*cysA*, *cysD*, *cysH*, *cysT*, *cysW* and *spb*) involved in sulfur metabolism was discovered. The actual stress response mechanism of this gene cluster remained to be characterized. Interestingly, *phzA2* gene involved in the production of virulence factors, phenazine, was down regulated with CHIR-090 treatment. [189] This suggested that the compound might have the ability to reduce virulence of the bacteria.

In the second part of this study, we treated wild type PAO1 with sub-inhibitory concentration of CHIR-090 for 21 days and generated three resistant populations of 090-TJH with MIC ranging from 16 μ g/ml to 32 μ g/ml. Three populations of 090-TJH, were sequenced to identify any mutated gene that might confer resistance against CHIR-090.

The reason we decided to sequence the population instead of a single colony is so that we can identify all possible mutations in the population. During the evolution assay, there is more than one type of mutation existing in the population. Hence, it is important to capture all the mutations within the population at the initial stage to provide a comprehensive insight into the convergent evolution events. Subsequently, we can then use PCR methods to verify the mutations present in a single colony isolated from that population.

Based on our preliminary analysis, there was a total of twenty mutations from the three populations. We further refined the mutation list to six genes that might be associated with antimicrobial resistance. These mutated genes were classified into three categories based on their functionality, being efflux pump, cell membrane/fatty acid/lipopolysaccharide synthesis and motility respectively.

Among the six mutated genes, *mexR* was the only common gene mutated in the three populations. This suggested that *mexR* gene might be responsible for the primary resistance mechanism against LpxC inhibitor, CHIR-090. *mexR* is a multidrug transcriptional repressor of the *mexAB-oprM* efflux pump. [190] A study by Chen and co-workers showed that the *mexR* regulator used an oxidation-sensing mechanism to mediate its resistance mechanism in *P. aeruginosa*. [191] Bactericidal antibiotics were found to stimulate the production of hydroxyl radical. Once generating sufficient oxidative stress, it can lead to cell death. [192] Given that CHIR-090 is a bactericidal antimicrobial, this might be one of the mechanisms that trigger the activation of *mexR*

and result in the activation of the efflux pump. It is notable that CHIR-090 is also a substrate of *mexAB-orpM* efflux pump. [153]

Next group of mutated genes plays an important role in the cell membrane synthesis. *murB* gene encodes for an UDP-N-acetylenolpyruvoylglucosamine reductase, which reduces UDP-GlcNAC-enolpyruvate to UDP-MurNAC in the peptidoglycan biosynthesis.[193] *pyrG* encodes for cytidine triphosphate (CTP) synthase which catalyzes the synthesis of the nucleotide cytidine triphosphate, which then activates 3-*dexoy-D-manno-octulosonic acid* (Kdo) before its transfer to lipid A. [194] Lastly, *fabF_I* encodes for 3-oxocayl-ACP synthase, which catalyzes the last step in the synthesis of unsaturated fatty acid by condensing acyl-ACP intermediate with malonyl-ACP. [195, 196]

According to the sequencing result, *murB* mutation was detected in two of the populations. This indicated the *murB* might play a major role in conferring resistance against CHIR-090. No study has been conducted so far to investigate the mechanism of *murB* in conferring resistance specifically to CHIR-090 in *P.aeruginosa*. However, a study found that *murB* gene played a role in providing resistance to antibiotics targeting the cell wall of *Bacillus subtilis*. [197] More in-depth studies are required to investigate on whether *murB* confers similar resistance in *P.aeruginosa*. Mutation of *fabG* gene resulted in the alteration of the fatty acid biosynthesis and conferred resistance against CHIR-090. [153] Since *fabF* gene is part of the fatty acid biosynthesis locus, it would be reasonable to infer that mutation of *fabF* gene would lead to similar resistance against

CHIR-090. Lastly, *pyrG* gene seemed to contribute to the resistance to CHIR-090 through modification of lipid A, but this still remains to be characterized.

Finally, the last group of gene mutated is involved in flagellar development and motility. *morA* regulates biofilm formation and is commonly associated with cystic fibrosis-related infections. [175, 198] Mutation of the gene might lead to increased biofilm formation resulting in enhanced resistance against CHIR-090. However, further studies would be required to confirm this hypothesis. Given that *fliF* is essential for flagellar assembly, mutation of the gene might lead to impaired motility, increasing the possibility of biofilm formation and decreasing susceptibility to CHIR-090. [174] Further work is warranted to verify our hypothesis.

In conclusion, the preliminary data from both transcriptomic and genomic analyses pointed to a similar observation that efflux pump and cell membrane alteration are the primary mode of resistance against CHIR-090. These observations were consistent with other previous studies [153, 154] However, it is important to note that our genomic analysis is only preliminary, and not comprehensive due to the lack of an experimental control group that should be treated by DMSO. There is a possibility that some of the mutations are caused by the effect of DMSO. As for gene related to efflux pump or fatty acid synthesis, we hypothesized that there is still a high chance that the mutations are driven by CHIR-090 treatment given that previous studies had reported similar findings. [153,154] Nevertheless, it is crucial to repeat the experiment with appropriate control to obtain a conclusive finding. Finally, it will be insightful to repeat similar analyses on

PAO1 biofilms to find out whether a similar trend is observed in biofilms condition. Such information will be useful for deciphering effective antimicrobial combination treatment since biofilm is known to have higher antibiotic resistance than the planktonic cells. [10]

Chapter 3: *In vitro* and *in vivo* efficacy of LpxC inhibitor, CHIR-090, alone or combined with colistin against *Pseudomonas aeruginosa* biofilms

Infections caused by Gram-negative bacteria, including *P. aeruginosa*, are increasingly becoming harder to treat with existing antimicrobials. [199] Being a versatile pathogen, *P. aeruginosa* plays an important role in a broad range of infections, including those that are biofilm associated. Biofilm-associated infections are particularly problematic because biofilm-embedded cells are more resistant to antibiotic treatments than their planktonic counterparts and able to develop to chronic infections. [200] When multidrug-resistant Gram-negative bacteria are involved, colistin often remains the only available therapeutic option. [201, 202]

Colistin (or polymyxin E) is a natural antibiotic that interacts with lipopolysaccharide (LPS) molecules in the outer membrane of Gram-negative bacteria, causing increased cell membrane permeability and leakage of cell contents, eventually leading to cell death. [203] In recent years, increasing numbers of *in vitro* and *in vivo* studies have reported on the emergence of colistin resistance. [204-209] With practically no new antibiotics being introduced on the market, this underlines the need for effective combinatorial treatment strategies involving compounds with new modes of action.

In the past decade, a lipid membrane enzyme, LpxC, was identified to be a potential and attractive target for antibiotic development. [210] LpxC is a zinc-dependent enzyme that

catalyzes the deacetylation of UDP-3-O-acyl-GlcNAc, the first step of lipid A biosynthesis. [211] Lipid A is the membrane anchor for LPSs and an essential component of the outer membrane of Gram-negative bacteria that protects cells from external agents [211] So far, several LpxC inhibitors, such as L-161,240, TU-514, BB-78485, and CHIR-090, have been identified and extensively characterized. [210, 211-213] Among all these inhibitors, CHIR-090 was the first known LpxC inhibitor that was shown to be active against *P. aeruginosa* and exhibited the most potent inhibitory effect against a wide range of LpxC enzymes with a subnanomolar affinity. [211] This compound demonstrated antibacterial activity similar to that of ciprofloxacin against *Escherichia coli* and *P. aeruginosa*. [214] However, the efficacy CHIR-090 alone or in combination against biofilms has not yet been examined.

Combination therapy is a powerful but also controversial strategy in treating infectious diseases, which aim to extend the antimicrobial spectrum of activity of each compound, enhance the bactericidal effect, and reduce the risk of the emergence of resistance. We hypothesized that colistin and CHIR-090 may work synergistically in eradicating bacterial biofilms through a self-promoted uptake mechanism, where colistin, as a cationic peptide, causes distortion of the outer membrane, thus facilitating the uptake of CHIR-090. [215] Hence, in our study, we aimed at evaluating the antimicrobial synergy of colistin in combination with CHIR-090 against both colistin-susceptible and -resistant *P. aeruginosa* biofilms using an *in vitro* susceptibility test and *in vivo* mouse implant model.

3.1 Materials and Methods

3.1.1 Bacterial strains and media

Two colistin-resistant clinical isolates of *P aeruginosa*, SCV-1 (CF173) and SCV-2 (CF273) described previously [216], the wildtype strain, PAO1, PAO1 strain tagged with GFP protein and its colistin-resistant mutant, PAO1-TJH, were tested in this study. PAO1-TJH was generated by successive exposure to increased colistin concentrations for 7 days. Muller-Hinton Broth (MNB, DifcoTM, Fisher Scientific, SAS, Illkirch, France) was used for *in vitro* experiments except for the flow cell experiments where ABT minimum medium supplemented with 0.5% glucose was employed. Luria-Bertani Agar, Miller (LB Agar, Miller, DifcoTM, Sparks Glencoe, USA) was used in the colistin population analysis profiles and colony counting.

3.1.2 Antimicrobial compounds

Colistin sulfate and CHIR-090 were commercially obtained from Sigma-Aldrich, France, and MedChem Express, NJ, USA respectively. Each compound was freshly prepared in water for colistin or dimethylsulfoxide, (DMSO) for CHIR-090 according to the Clinical and Laboratory Standard institute, (CLSI) guidelines. [217]

3.1.3 Population analysis profiles, PAP

The presence of colistin resistant subpopulations or colistin heteroresistance was examined in PAO1 according to the protocol described elsewhere with some modifications. [208] An initial inoculum of 10⁹ CFU/ml was prepared in sterile normal saline using an overnight culture grown in LB. Serial dilutions of this inoculum were

then plated onto LB agar plates containing concentrations of colistin and CHIR-090 ranging from 0 to 16 or 0 to 2 µg/ml, respectively. Plates were incubated for 36 hours at 37°C. Colony forming unit per ml (CFU/ml) were plotted versus time using PRISM 7.0 (GraphPad Software, Inc., San Diego, CA, USA). Experiment was performed in duplicate to ensure reproducibility. Heterogeneous resistance to colistin was defined as the presence of detectable subpopulations growing at concentrations greater than the MIC, as described. [218]

3.1.4 Antimicrobial susceptibility

Minimum Inhibitory Concentrations (MIC) values of CHIR-090 and colistin were determined for each tested *P. aeruginosa* strain according to the CLSI guidelines using a starting inoculum of $\sim 10^6$ CFU/ml. [217] Potential for synergy of colistin combined with CHIR-090 was examined against all tested strains using checkerboard assay and concentrations ranging from 0 to 64 and 0 to 16 µg/ml, for colistin and CHIR-090, respectively. [219] Bacterial growth was assessed visually after 18 hours incubation at 37°C. Σ FIC was then calculated by addition of FIC A to FIC B (FIC A + FIC B) where FIC A is the minimum inhibitory concentration (MIC) of drug A in combination/MIC of drug A alone, and FIC B is the MIC of drug B in combination/MIC of drug B alone. Synergistic activity was interpreted using the index of fractional inhibitory concentration (Σ FIC) as follow: Σ FIC<0.5: synergy, $0.5<\Sigma$ FIC<4: no interaction and Σ FIC> 4: antagonism. [220] All the experiments were repeated three times.

3.1.5 Antimicrobial susceptibility of biofilm embedded cells (MBEC)

Minimum Biofilm Eradication Concentration (MBEC) values of colistin and CHIR-090 were evaluated in triplicates against *P. aeruginosa* biofilms, as described by Naparstek et al. [221] Briefly, biofilms were grown in 96-wells microplate for 24 hours at 37⁰C in MH medium and then washed twice with 0.9% NaCl to remove planktonic cells. Attached biofilms were then subjected to combination of different antimicrobial concentrations in a checkerboard assay format. Antimicrobial concentrations tested varied from 0 to 512 µg/ml and 0 to 128 µg/ml for colistin and CHIR-090, respectively. Plates were incubated for 24 hours at 37⁰C. After antimicrobial challenge, wells were thoroughly washed with 0.9% NaCl to remove any antimicrobial residue. Fresh 200 µl of MH growth medium were added to the washed wells and then incubated for 24 hours at 37⁰C. MBEC values were determined as the lowest antibiotic concentrations that prevented bacterial growth from treated biofilm. Potential for synergy was also evaluated by calculating the index of fractional inhibitory concentration (ΣFIC) from the resulting biofilm checkerboard assay. All experiments were performed in triplicate.

3.1.6 Bead biofilm assay

The effect of colistin and CHIR-090 combination was evaluated using bead biofilms assay as described by Konrat et al. [222] Diluted (OD₆₀₀ = 0.002) overnight PAO1 culture was added to each well of a 24 macroplate containing 3mm glass beads (EMD Millipore Corporation, Merck, Darmstadt, Germany) immersed into 1ml of MH medium. Biofilm of PAO1 was allowed to grow at 37⁰C for 72 hours with fresh medium replenished every 24 hours. Beads were then washed with 0.9% NaCl to remove

planktonic cells and loose biofilm and immersed into fresh MHII medium. The following treatments were tested: DMSO (0.0005%), colistin (at 5 and 10 µg/ml) and CHIR-90 (at 4 µg/ml) alone or combined with colistin (at 5 and 10 µg/ml). After 24 hours treatment, bead were removed with sterile forceps at 0, 4, 8, and 24 h and placed into eppendorf tubes containing 0.9% NaCl. Biofilm cells were recovered following 4 alternating cycles of vortexing (10 sec) and sonication (37Hz). The samples were serial diluted, spotted onto LB agar plates and then incubated at 37⁰C for 16 hours. All experiments were conducted in triplicate.

3.1.7 Biofilms in flow cell chambers

Biofilms of *gfp*-tagged *P. aeruginosa* strains were cultivated in a well described 40 x 4 x 1mm three channels flow cells chamber (Biocentrum-DTU). The flow chamber was set up according to a previous described protocol [223] and filled with ABTG medium. The flow channels were each inoculated with 500 µl of 1:1000 x diluted overnight culture and then left incubated for 1 hour without any media flow. Medium flow was initiated using the Cole-Palmer peristaltic with a flow velocity of 0.2 mm/s. Three replicate channels were applied for making triplicates. After 72 hours incubation in the flow cells, the flow cells were treated with the following regimens; DMSO (0.0005%), colistin (at 5 and 10 µg/ml) and CHIR-090 (at 4 µg/ml) alone or combined with colistin (at 5 and 10 µg/ml). After 24 hours of antimicrobial exposure, 200 µl of 20 µM of propidium Iodine (PI, Sigma-Aldrich) were injected into each individual flow channels to stain dead cells of biofilm. Microscopic images were captured and acquired using Zeiss LSM 780 confocal laser scanning microscope (Carl Zeiss, Germany) with a 20 X objective for

monitoring GFP and PI fluorescence. 10 images from each sample were used to calculate the dead to live ratio using Imaris 7.7 (Bitplane AG, Switzerland) and the mean values of the dead to live ratio of sample were presented.

3.1.8 The mouse biofilm implant model of infection

Experiments with this model were performed by Dr. Chua Song Lin, Dr. Joey Yam and Mrs. Mary Salido according to the NACLAR Guidelines and Animal and Birds (Care and Use of Animals for scientific purpose) Rules by Agri-Food & Authority of Singapore (AVA), with authorization and approval by the Institutional Care and Use Committee (IACUC) and Nanyang Technological University, under the permit number A-0191. Seven weeks-old female BALB/c mice (Taconic M&B A/S) were used in this study. The experimental preparation and setup was based on the protocol described by Song et al. [224] In summary, PAO1 biofilms were grown on cylindrical implants (3mm x 5mmØ) in 0.9% NaCl at 37°C with shaking at 110 rpm for 20 hours. After incubation, biofilm-coated implants were washed three times with 0.9% NaCl and transplanted into the peritoneum of anesthetized mice. Antibiotic regimens were injected as a single dose at the implantation site of groups of five mice after one-day incubation. Treatments and associated groups included: Control treated with no antibiotic (injection of x ml of DMSO), test group 1 treated with 10mg kg⁻¹ colistin (which is well below lethal dose of 86 mg kg⁻¹ in mice), test group 2 treated with 4mg kg⁻¹ CHIR-090 and test group 3 treated with 10mg kg⁻¹ colistin and 4mg kg⁻¹ CHIR-090. After 24 hours, mice were euthanized; implants were collected from the peritoneum and washed with 0.9% NaCl. The implants were then sonicated in ice-filled water sonicator using an Elmasonic

P120H (Elma, Germany) at 50% power and 37 KHz for 10 minutes followed by vortexing the samples three times for 10 seconds. Spleens were also collected and homogenized using the Bio-Gen PRO200 Homogenizer (Pro Scientific, US) at maximum power on ice. Implant and spleen samples were then serially diluted in 0.9% NaCl, plated onto LB agar and incubated overnight at 37°C. Colony forming units (CFU) were calculated and plotted versus treatment using PRISM 7.0 (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as means $\pm$ s.d.

3.1.9 Statistical analysis.

The killing effects of each antimicrobial regimen tested *in vitro* were analyzed using t-test. All statistical analyses were performed using PRISM 7.0 (GraphPad Software, Inc., San Diego, CA, USA). A P value of <0.05 was considered significant.

3.1.10 Experimental Workflow

3.2 Results

3.2.1 Antimicrobial susceptibility under planktonic conditions

MIC values of colistin and CHIR-090 are reported in Table 3.1. PAO1 exhibited MIC values of 1 and 0.5 $\mu\text{g/ml}$ for colistin and CHIR-090, respectively (Table 3.1). Interestingly, the *in vitro* selected colistin-resistant derivative of the PAO1 strain was more susceptible to CHIR-090 ($\text{MIC}_{\text{CHIR-090}} = 0.25\mu\text{g/ml}$) as compared to its parental

strain (Table 3.1). The two clinical isolates, SCV (1) and SCV (2) exhibited reduced susceptibility colistin with MIC values of 16 and 8 µg/ml, respectively, but were highly susceptible to CHIR-090 with MIC values of 0.0625 µg/ml, (ie 8 fold lesser than PAO1) (Table 3.1).

Strains	MIC (µg/ml)		Σ FIC (CST + CHIR-090)*
	CST	CHIR-090	
PAO1	1	0.5	0.75 (0.5 + 0.25)
PAO1-TJH	64	0.25	0.13(1 + 0.0625)
SCV (1)	16	0.0625	0.5 (4 + 0.015)
SCV (2)	8	0.0625	0.75 (4 + 0.015)

Table 3.1: *In-vitro* susceptibility to colistin (CST) and CHIR-090 alone or in combination of the 4 tested *P. aeruginosa* strains. * Concentrations of CST and CHIR-090 for the reported Σ FIC values.

Wild type strain, PAO1 demonstrated heterogeneous resistance to both colistin and CHIR-090 with subpopulations growing at concentrations equivalent to 4 x MIC (4 and 2 mg/L for colistin and CHIR-090, respectively) (Figure 3.1). Interestingly, when CHIR-090 (at 0.25 mg/L = 0.5 MIC), was added to colistin, we observed a population shift towards lower colistin MIC values with subpopulation growing at concentrations up to 2 x MIC only (Figure 3.1), suggesting that the drug combination has a potential for

synergy. Potential for synergy of colistin combined with CHIR-090 was further examined by checkerboard assay. Synergistic effect of colistin combined with CHIR-090 was confirmed for two colistin resistant isolates, PAO1-TJH and SCV (1) ($\Sigma\text{FIC} \leq 0.5$) but no interaction against PAO1 and SCV (2) ($\Sigma\text{FIC} > 0.5$). (Table 3.1)

Figure 3.1: Colistin population analysis profiles of PAO1 using colistin monotherapy, CHIR-090 monotherapy and colistin-CHIR-090 combination. 10⁸ CFU of PAO1 were plated on LB plates containing 0 µg/ml to 8 µg/ml of colistin alone (black line), 0 µg/ml to 2 µg/ml CHIR-090 alone (dashed line) or 0 µg/ml to 8 µg/ml of colistin with 0.25 µg/ml (1/4 MIC) of CHIR-090 (dotted line).

3.2.2 Antimicrobial susceptibility of *P. aeruginosa* biofilms

The colistin minimum biofilm eradication concentration (MBEC) values were significantly higher than the MIC values (256 µg/ml for PAO1, SCV-1, and SCV-2 and 512 µg/ml for PAO1-TJH) (Table 3.2). In contrast, CHIR-090 exhibited lower MBEC values against all tested strains, with the values ranging from 8 to 128 µg/ml (Table 3.2).

Interestingly, using a checkerboard assay and biofilm conditions, synergy (Σ FIC range, 0.13 to 0.31) was observed against both colistin-susceptible and -resistant *P. aeruginosa* biofilms. Of note, biofilm eradication could be achieved at lower concentrations for SCV-1 than for the other strains (2 and 1 μ g/ml of colistin and CHIR-090, respectively).

Strains	MBEC (μ g/ml)		Σ FIC (CST + CHIR-090)*
	CST	CHIR-090	
PAO1	256	>128	0.34 (64 + 1)
PAO1-TJH	512	64	0.34(64 + 16)
SCV (1)	256	8	0.13 (2 + 1)
SCV (2)	256	128	0.31(64 + 8)

Table 3.2: *In-vitro* MBEC values of CST and CHIR-090 alone and in combination against 4 *P. aeruginosa* strains. * Concentrations of CST and CHIR-090 for the reported Σ FIC value.

3.2.3 Colistin and CHIR-090 efficacy against PAO1 biofilms grown in a biofilm flow cell model.

The efficacy of colistin and CHIR-090 combination against 3-day-old biofilms of PAO1

was evaluated in a flow cell chamber. When dimethyl sulfoxide (DMSO; used as a control) was added, a minimal killing effect was observed (Figure 3.2Ai). CHIR-090 monotherapy at 4 $\mu\text{g/ml}$ failed to eradicate the metabolically inactive layer of the biofilm, and only the metabolically active population was affected (Figure 3.2Aii). Using colistin at 5 $\mu\text{g/ml}$, a killing effect was observed only at the bottom layer of the biofilm, as indicated by the increase in the propidium iodide (PI) fluorescence (Figure 3.2Aiii). Treatment with colistin at 10 $\mu\text{g/ml}$ resulted in a much higher level of bacterial eradication. The results for the remaining populations observed in the flow cells support the population analysis profiles (PAP), suggesting the presence of a colistin-tolerant subpopulation (Figure 3.2Av). Combination of colistin at 10 $\mu\text{g/ml}$ with CHIR-090 allowed for the complete eradication of these subpopulations with higher colistin MIC (Figure 3.2Avi) Interestingly, by combining colistin at 5 $\mu\text{g/ml}$ with CHIR-090 at 4 $\mu\text{g/ml}$, an increase in the killing effect was observed on the top layer of the biofilm (Figure 3.2Aiv). These microscopic observations were supported by the evaluation of the killing efficacy of the different treatment regimens using the dead/live ratio (Figure 3.2B)

Figure 3.2: *In vitro* efficacy of colistin and CHIR-090 alone or in combination against 3-day-old PAO1 biofilms in biofilm flow cells. (A) Confocal imaging of the killing effects of DMSO (control) (i), CHIR-090 at 4 µg/ml (ii), colistin at 5 µg/ml (iii), colistin at 5 µg/ml combined with CHIR-090 at 4 µg/ml (iv), colistin at 10 µg/ml (v), and colistin at 10 µg/ml combined with CHIR-090 at 4 µg/ml (vi). Bars = 50 µm. (B) Dead-to-live ratio of colistin and CHIR-090 alone and in combination. Statistical significance was analyzed by Student's t test (***, P < 0.01)

Lastly, we also used a bead biofilm assay to confirm the synergistic effect of colistin and CHIR-090 by direct CFU counts, and similar results obtained (Figure 3.3).

Figure 3.3: Evaluation of colistin and CHIR-090 combination using beads PAO1 biofilm assay. Raw bacteria count for the killing effect of i) DMSO (Control), ii) CHIR-090 at 4 µg/ml, iii) colistin at 5 µg/ml, iv) colistin at 5 µg/ml combined with CHIR-090 at 4 µg/ml, v) colistin at 10 µg/ml and vi) colistin at 10 µg/ml combined with CHIR-090 at 4 µg/ml. Statistical significance were analyzed with Student's T-test (* $P < 0.05$). N.D – Not detectable

3.2.4 *In vivo* evaluation of colistin and CHIR-090 efficacies, alone or in combination, against PAO1 biofilms grown in a mouse implant model of infection.

The *in vivo* efficacy of colistin and CHIR-090, alone and in combinations was evaluated using a mouse implant model of infection. The results are presented in Figure 3.4. Similar log counts were observed for the mice from the control group (treated with DMSO) and the untreated group (treated with 0.9% NaCl) in both the implant and the spleen (Figure 3.4). For both the implant and spleen, CHIR-090 monotherapy resulted in a decreased CFU counts up to 2- $\log_{10}$ folds, whereas colistin caused a 3- $\log_{10}$ reduction in CFU counts compared to that in the untreated or control group (Figure 3.4). The combination of colistin and CHIR-090 resulted in a greater reduction in the number of CFU up to 4 $\log_{10}$ folds (Figure 3.4).

Figure 3.4: *In vivo* evaluation of colistin (10 $\mu\text{g}/\text{ml}$) and CHIR-090 (4 $\mu\text{g}/\text{ml}$) alone and in combination using a mouse implant model of PAO1 biofilm. Each experimental group included five replicates. Credit to Dr. Chua Song Lin, Dr. Joey Yam and Mrs. Mary

Salido for performing the *in vivo* mouse implant experiment

3.3 Discussions

Colistin, also known as polymyxin E, is a cationic peptide often used in combination therapy as the last-resort option to treat such infections. [201] However, due to its extensive use, bacterial strains with reduced susceptibility to colistin have begun to emerge in clinical settings. [204-207]

Combination therapy is used empirically to (i) ensure that at least one of the agents is active against the responsible pathogen, (ii) aim for potential synergy and therefore enable faster killing, and (iii) mitigate the development of resistance during treatment. As a result, this strategy is recognized to increase the likelihood of faster clearance of the infection and, therefore, recovery of the patients.

Researchers have been interested in the microbiological and therapeutic effects of the combination of colistin with selected antimicrobials over the last 2 decades, and such combinations have been well studied. [209] This combination treatment strategy has shown encouraging results; however, well-designed clinical studies supporting the *in vitro* and *in vivo* observations obtained with new antimicrobial compounds in combination with colistin are still warranted. With respect to bacterial biofilms, LpxC inhibitors, such as CHIR-090, have drawn recent attention. These compounds target the LpxC enzyme, involved in lipid A (the anchor membrane for LPS) synthesis. While CHIR-090 has previously been demonstrated to be highly effective against planktonic cells of *P. aeruginosa*, this class of antibiotic has not been examined for its antibiofilm

properties. [214] Given that colistin and CHIR-090 target different components of the LPS of Gram-negative bacteria, we hypothesized that there might be a potential for synergy when combining colistin and CHIR-090.

Of interest and as never been reported before, we did not observe cross-resistance between colistin and the tested LpxC inhibitor. Indeed, CHIR-090 demonstrated low MIC values (less than 1 µg/ml) against both colistin-susceptible and -nonsusceptible *P. aeruginosa* strains. The combination of colistin with subinhibitory concentrations of CHIR-090 also resulted in a decrease in the colistin MICs of the PAO1 population, suggesting that (i) CHIR-090 may have the potential to eradicate PAO1 subpopulations with lower colistin susceptibility and (ii) the combination may demonstrate synergistic effects against *P. aeruginosa*. This is of particular importance, since conventional dosing of antibiotics most likely selects for the more resistant subpopulations if highly resistant subpopulations of a heteroresistant isolate are not considered. The combination of colistin with CHIR-090 is therefore less likely to select for colistin resistance over time. Using both static and flow cell conditions modes of growth, a synergistic effect against all tested strains grown as planktonic and biofilm cells was observed. As we hypothesized, the effect was greater when cells were grown as a biofilm than when they were grown in the planktonic state. The combination of CHIR-090 and colistin also exhibited a stronger bactericidal effect against colistin-nonsusceptible strains than colistin-susceptible strains. Altogether, these observations support a potential mechanism by which CHIR-090 targets subpopulations with decreased colistin MIC values, with a greater impact on cells growing in biofilms.

To gain a better spatial understanding of the killing dynamics of colistin and CHIR-090, we tested the combinatorial treatment in a biofilm flow cell system. On the basis of our observations, CHIR-090 seems to target metabolically active cells. This might be due to the favorable phenotype of cells on the top layer of the biofilm to CHIR-090 activity or the reduced penetration ability of CHIR-090 across the biofilm matrix. [225] In contrast to what has been reported previously, colistin targeted the subpopulation located at the substratum of the biofilm. [226] This observation was associated with the migration of the tolerant subpopulation at the top layer of the biofilm exposed to colistin. [226] These distinct killing dynamics of colistin and CHIR-090 support our initial hypothesis and findings that the combination of the two antimicrobials has the potential to be synergistic against a *P. aeruginosa* biofilm by targeting different subpopulations. Interestingly, this effect was observed with subinhibitory concentrations of colistin that are also clinically achievable. *In vivo* animal experiments supported the *in vitro* observations, which the combination of colistin and CHIR-090 is more effective than monotherapy. If the effect of the combination did not result in total eradication of the biofilms, it is important to keep in mind that we tested only a single-dose regimen. The administration of repeated dosages over a course of 5 to 7 days, which would be used in practice, would provide us with a better prognosis of the potential therapeutic outcomes of such a combined treatment. The testing of additional regimens, including those using clinically relevant dosages based on the area under the concentration-time curve/MIC parameter, is now warranted to confirm these preliminary findings and further evaluate the therapeutic interest in colistin combined with CHIR-090. Finally, and of great interest, the combined

treatment contributed to control of the spread of the bacteria to the spleen.

In conclusion, our study demonstrated for the first time the *in vitro* and *in vivo* antimicrobial efficacies of CHIR-090 alone and in combination with colistin against biofilms of both colistin-susceptible and -nonsusceptible *P. aeruginosa*. While our data suggested that CHIR-090 has a limited ability to eradicate *P. aeruginosa* biofilms by itself, the compound seems to be capable of suppressing colistin-tolerant subpopulations.

A better understanding of the molecular mechanisms responsible for the reduced colistin susceptibility in the subpopulations of each strain with higher colistin MICs is now of great interest to better understand the mechanism of synergy observed with CHIR-090. Inactivation of lipid A biosynthesis has already been reported to be associated with reduced susceptibility to colistin in *Acinetobacter baumannii* and recently in *P. aeruginosa*. [227, 228] Of interest, a complex and reversible mechanism involving the regulatory network, genetic alterations, and lipid A biosynthesis was suggested in *P. aeruginosa*. [229] These findings highlight the great interest in inhibitors of lipid A biosynthesis and their therapeutic potential in combination with colistin for the treatment of Gram-negative bacterial infections associated or non-associated with biofilms. In-depth studies are therefore required to (i) elucidate the mechanisms of synergy and (ii) investigate the therapeutic potential of CHIR-090 in combination with colistin. Nevertheless, our results suggest the strong potential of CHIR-090 or other LpxC inhibitors to be used adjunct treatments to prevent the emergence of colistin resistance during therapy. [230, 231]

Chapter 4: *In vitro* and *in vivo* anti-biofilm efficacy of CHIR-090 combined with different classes of antimicrobials against *Pseudomonas aeruginosa* biofilms

Opportunistic pathogen *P. aeruginosa* is the leading cause of morbidity and mortality in cystic fibrosis patients. [232] *P. aeruginosa* infection is difficult to eradicate due to its ability to form biofilms that lead to higher antimicrobial resistance. [233] In biofilms, bacterial community produces an extracellular matrix to protect itself against antimicrobial therapy and host immune system. [234] Although the chronic infections can be mitigated by aggressive antimicrobial therapy, it remains largely ineffective in total eradication. With limited therapeutic options available, it will be worthwhile to explore other possible effective anti-biofilms antimicrobial combinations.

In the previous chapters, we investigated the effectiveness of colistin and CHIR-090 combination against *P.aeruginosa* biofilms. While the combination had shown to be effective against PAO1 biofilms, colistin has been known for its renal toxicity. [235] Long-term use of colistin may not be beneficial to patients with renal impaired condition and may further complicate the condition of the patient. [236] Also, the use of colistin is usually considered as the last resort of the treatment, and if possible, other safer options would be preferred. [237]

In this chapter, we evaluated the anti-biofilm ability of CHIR-090 combined with different classes of antimicrobials using similar *in vitro* and *in vivo* methods. These

antibiotics are carbenicillin (β -lactams), ciprofloxacin (fluoroquinolones), tobramycin (aminoglycosides) and azithromycin (macrolide).

Ciprofloxacin and tobramycin are common antibiotics used to treat *P. aeruginosa* biofilms. In previous studies, these two antibiotics combined with colistin demonstrated enhanced *in vitro* and *in vivo* potency against *P. aeruginosa* biofilms. [66, 85] Given that CHIR-090 and colistin share similar mechanism of action by targeting the synthesis of lipid A, we hypothesized that similar synergy effect might be achieved with tobramycin and ciprofloxacin. Another class of antibiotics that we will be studying in the combination with CHIR-090 is azithromycin. *In vitro* studies showed azithromycin inhibits biofilm growth, alginate production and synthesis of other virulence factors via interfering with the quorum sensing (QS) system. [86, 238] Furthermore, azithromycin is widely prescribed as a maintenance therapy in *P. aeruginosa* infections in cystic fibrosis patients and demonstrated clinical benefits to the patients. [239] Given azithromycin's anti-biofilm activity, we decided to evaluate the possible synergistic activity of azithromycin and CHIR-090 combination against *P. aeruginosa* biofilms. The last class of antibiotics that we evaluate is carbenicillin (β -lactams). Carbenicillin is commonly used to treat *P. aeruginosa* infections and is effective in treating urinary tract infections. [240] Hence, we would like to study whether carbenicillin and CHIR-090 combination would have increased potency against *P. aeruginosa* biofilms.

4.1 Materials and Methods

4.1.1 Bacterial strains and media

Wildtype strain, PAO1, PAO1 tagged with GFP, its CHIR-090 resistant derivative, 090-TJH and its tobramycin resistant derivative, Tobra-TJH were tested in this study. CHIR090-TJH and Tobra-TJH were generated by successive exposure to sub-minimum inhibitory concentration of CHIR-090 and tobramycin for 21 days. Muller-Hinton Broth (MNB, Difco™, Fisher Scientific, SAS, Illkirch, France) was used for *in vitro* experiments except for the flow cell experiments where ABT minimum medium supplemented with 0.5% glucose was employed. Luria-Bertani Agar, Miller (LB Agar, Miller, Difco™, Sparks Glencoe, USA) was used for colony counting.

4.1.2 Antimicrobial compounds

Tobramycin, azithromycin, carbenicilin, ciprofloxacin and CHIR-090 were purchased from Sigma-Aldrich, France, and MedChem Express, NJ, USA respectively. Each compound was freshly prepared in water for tobramycin, carbenicilin and ciproflaxcin; ethanol for azithromycin or dimethylsulfoxide, (DMSO) for CHIR-090 according to the Clinical and Laboratory Standard institute, (CLSI) guidelines. [217]

4.1.3 Antimicrobial susceptibility

Minimum inhibitory concentrations (MIC) values of CHIR-090, tobramycin, ciproflaxcin, azithromycin and carbenicilin were determined for tested *P. aeruginosa*, PAO1 strains according to the CLSI guidelines using a starting inoculum of $\sim 10^6$ CFU/ml. [217] Potential for synergy of all antibiotic combined with CHIR-090 was examined against all tested strains using checkerboard assay and concentrations ranging

from 0 to 64 and 0 to 16 µg/ml, for tobramycin, ciprofloxacin, azithromycin, carbenicilin and CHIR-090, respectively. [219] Bacterial growth was assessed visually after 18 hours incubation at 37⁰C. Σ FIC was then calculated by addition of FIC A to FIC B (FIC A + FIC B) where FIC A is the minimum inhibitory concentration (MIC) of drug A in combination/MIC of drug A alone, and FIC B is the MIC of drug B in combination/MIC of drug B alone. Synergistic activity was interpreted using the index of fractional inhibitory concentration (Σ FIC) as follow: Σ FIC>0.5: synergy, 0.5< Σ FIC<4: no interaction and Σ FIC> 4: antagonism. [220]

4.1.4 Antimicrobial susceptibility of biofilm embedded cells (MBEC)

Minimum biofilm eradication concentration (MBEC) values of tobramycin, azithromycin, ciproflaxcin, carbenicilin and CHIR-090 were evaluated in triplicates against *P. aeruginosa* biofilms, as described by Naparstek et al. [221] Briefly, biofilms were grown in 96-wells microplate for 24 hours at 37⁰C in MH medium and then washed twice with 0.9% NaCl to remove the planktonic cells. Attached biofilms were subjected to combination of different antimicrobial concentrations in a checkerboard assay format. Antimicrobial concentrations tested varied from 0 to 512 µg/ml and 0 to 192 µg/ml for the four different antibiotics and CHIR-090, respectively. Plates were incubated for 24 hours at 37⁰C. After antimicrobial challenge, wells were thoroughly washed with 0.9% NaCl to remove any antimicrobial residue. Fresh 200 µl of MH growth medium were added to the washed wells and incubated for 24 hours at 37⁰C. MBEC values were determined as the lowest antibiotic concentrations that prevented bacterial growth from treated biofilm. Potential for synergy was also evaluated by calculating the index of

fractional inhibitory concentration (Σ FIC) from the resulting biofilm checkerboard assay. All experiments were performed in triplicate.

4.1.5 Bead biofilms time-kill assay

The killing dynamic of tobramycin and CHIR-090 combination was evaluated using bead biofilms assay as described by Konrat et al. [222] Diluted ($OD_{600} = 0.002$) overnight PAO1 culture was added to each well of a 24 microplate containing 3mm glass beads (EMD Millipore Corporation, Merck, Darmstadt, Germany) immersed in 1ml of MH medium. PAO1 biofilms was allowed to grow at 37°C for 72 hours with fresh medium replenished every 24 hours. Beads were then washed with 0.9% NaCl to remove planktonic cells and loose biofilm and immersed in fresh MHII medium. The following treatments were tested: DMSO (0.0005%), tobramycin (10 μ g/ml) and CHIR-090 (at 4 μ g/ml) alone or combined with tobramycin (10 μ g/ml). After 24 hours treatment, bead were removed with sterile forceps at 0, 4, 8, and 24 h and placed into 2ml sterile eppendorf tubes containing 0.9% NaCl. Biofilm cells were recovered following 4 alternating cycles of vortexing (10 sec) and water bath sonication (37Hz). The samples were serial diluted and spotted onto LB agar plates incubated at 37°C for 16 hours. Number of colony forming unit (CFU) was plotted against time. All experiments were conducted in triplicate

4.1.6 Biofilms in flow cell chambers

Biofilms of *gfp*-tagged *P. aeruginosa* strains were cultivated in a 40 x 4 x 1mm three channels flow cells chamber (Biocentrum-DTU). The flow chamber was set up according to a previous described protocol and filled with ABTG medium. [223] The flow channels

were each inoculated with 500 µl of 1:1000 x diluted overnight culture and left incubated for 1 hour without any medium flow. Medium flow was initiated using the Cole-Palmer peristaltic with a flow velocity of 0.2 mm/s. Three replicate channels were used for experimental condition. After 72 hours incubation in the flow cells, the flow cells were treated with the following regimens; DMSO (0.0005%), four different antibiotics (10 µg/ml for ciprofloxacin and tobramycin, 8 µg/ml azithromycin and 300 µg/ml carbenicilin) and CHIR-090 (at 4 µg/ml) alone or combined with the four different antibiotics (10 µg/ml for ciprofloxacin and tobramycin, 8 µg/ml azithromycin and 300 µg/ml carbenicilin). After 24 hours of antimicrobial exposure, 200 µl of 20 µM of propidium Iodine (PI, Sigma-Aldrich) were injected into each individual flow channels to stain dead cells of biofilm. Microscopic images were captured and acquired using Zeiss LSM 780 confocal laser scanning microscope (Carl Zeiss, Germany) with a 20 X objective for monitoring GFP and PI fluorescence.

4.1.7 Mouse biofilm implant model of infection

Experiments with this model were performed by Dr. Joey Yam and Mrs. Mary Salido according to the NAELAR Guidelines and Animal and Birds (Care and Use of Animals for scientific purpose) Rules by Agri-Food & Authority of Singapore (AVA), with authorization and approval by the Institutional Care and Use Committee (IACUC) and Nanyang Technological University, under the permit number A-0191. Seven weeks-old female BALB/c mice (Taconic M&B A/S) were used in this study. The experimental preparation and setup was conducted based on the protocol described by Song et al. [220] In summary, PAO1 biofilms were grown on cylindrical implants (3mm x 5mmØ)

in 0.9% NaCl at 37°C with shaking at 110 rpm for 20 hours. After incubation, biofilm-coated implants were washed three times with 0.9% NaCl and transplanted into the peritoneum of anesthetized mice. Antibiotic regimens were injected as a single dose at the implantation site of groups of five mice. Treatments and associated groups included: Control treated with no antibiotic (injection of 0.0002% of DMSO), test group 1 treated with 10 mg kg⁻¹ tobramycin, test group 2 treated with 4 mg kg⁻¹ CHIR-090 and test group 3 treated with 10 mg kg⁻¹ tobramycin and 4 mg kg⁻¹ CHIR-090. After 24 hours, mice were euthanized; implants were collected from the peritoneum and washed with 0.9% NaCl. The implants were then sonicated in ice-filled water sonicator using an Elmasonic P120H (Elma, Germany) at 50% power and 37 KHz for 10 minutes followed by vortexing the samples three times for 10 seconds. Spleens were also collected and homogenized using the Bio-Gen PRO200 Homogenizer (Pro Scientific, US) at maximum power on ice. Implant and spleen samples were then serially diluted in 0.9% NaCl, plated onto LB agar and incubated overnight at 37°C. Colony forming units (CFU) were calculated and plotted versus treatment using PRISM 7.0 (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as means ± s.d.

4.1.8 Statistical analysis

The killing effects of each antimicrobial regimen tested *in vitro* were analyzed using t-test. All statistical analyses were performed using PRISM 7.0 (GraphPad Software, Inc., San Diego, CA, USA). A P value of <0.05 was considered significant.

4.1.9 Experimental Workflow

4.2 Results

4.2.1 Antimicrobial susceptibility under planktonic condition

MIC values of tobramycin (TBR), ciprofloxacin (CIP), azithromycin (AZM), carbencillin (CARB) and CHIR-090 are reported in Table 4.1. PAO1 exhibited MIC values of 0.5 µg/ml for both tobramycin and ciprofloxacin, 64 µg/ml for azithromycin and for carbencillin and 1µg/ml for CHIR-090, respectively (Table 4.1). Potential for synergy of respective antibiotics combined with CHIR-090 was examined by checkerboard assay. Out of all antibiotics tested, synergistic effect was only observed in tobramycin combined with CHIR-090 against PAO1 ($\Sigma\text{FIC} \leq 0.5$). No synergistic interaction was observed with ciprofloxacin ($\Sigma\text{FIC} > 0.5$), azithromycin ($\Sigma\text{FIC} > 0.5$) and carbencillin ($\Sigma\text{FIC} > 0.5$) combination. (Table 4.1)

Antibiotic	(µg/ml)		ΣFIC
	MIC	CHIR-090 (MIC)	(Antibiotic + CHIR-090)*
Tobramycin (TBR)	0.5	1	0.5 (0.25 + 0.25)
Ciprofloxacin (CIP)	0.5	1	1.25 (0.5+0.5)
Azithromycin (AZM)	64	1	0.75 (16 + 0.5)
Carbencillin (CARB)	64	1	2 (64 + 1)

Table 4.1: *In-vitro* susceptibility to TBR, CIP, AZM and CARB and CHIR-090 alone or in combination of the *P. aeruginosa*, PAO1. *Concentrations of different antibiotics and CHIR-090 for the reported Σ FIC values.

4.2.2 Antimicrobial susceptibility of *P. aeruginosa* biofilms

The minimum biofilm eradication concentration (MBEC) values for each respective antibiotic and in combination with CHIR-090 were reported in Table 4.2. MBEC of tobramycin against PAO1 biofilms was 16 folds higher than its MIC value of 1 $\mu\text{g/ml}$ (Table 4.2). MBEC of ciprofloxacin was significantly higher (128 $\mu\text{g/ml}$) as compared to its MIC value of 1 $\mu\text{g/ml}$. Carbenicillin and azithromycin were ineffective against PAO1 biofilms with a MBEC more than 1024 $\mu\text{g/ml}$. As shown in the previous chapter, CHIR-090 exhibited MBEC of >128 $\mu\text{g/ml}$ against PAO1 (Table 4.2). Interestingly, using a checkerboard assay in biofilm conditions, strong synergy was observed for tobramycin-CHIR-090 combination against PAO1 biofilms with a ΣFIC value of 0.3125. On the other hand, ciprofloxacin-CHIR-090 combination showed that ciprofloxacin alone was sufficient to eradicate PAO1 biofilms with an MBEC of 128 $\mu\text{g/ml}$ while carbenicillin and azithromycin combined with CHIR-090 respectively did not demonstrate any synergy ($\Sigma\text{FIC}>2$).

Antibiotic	(µg/ml)		ΣFIC
	MBEC	CHIR-090 (MBEC)	(Antibiotic+ CHIR-090)*
Tobramycin (TBR)	16	>128	0.3125 (4 + 16)
Ciprofloxacin (CIP)	128	>128	1 (128 + 0)
Azithromycin (AZM)	>1024	128	1 (0 +128)
Carbenicillin (CARB)	>1024	>128	> 2 (Nil)

Table 4.2: *In-vitro* MBEC values of TBR, CIP, AZM, CARB and CHIR-090 alone and in combination against PAO1 biofilms. *Concentrations of different antibiotics and CHIR-090 for the reported ΣFIC value.

4.2.3 Different classes of antimicrobial and CHIR-090 combination efficacy against PAO1 biofilms grown in a biofilm flow cell model

The efficacy of the different antibiotics and CHIR-090 combination was evaluated against 3-days-old biofilms of PAO1 in a flow cell chamber. There was minimal killing of biofilms when dimethyl sulfoxide (DMSO; used as a control) was added (Figure 4.1(i)). Consistent with other previous studies, ciprofloxacin monotherapy at 10 µg/ml

affected only the metabolically active layer of the biofilms (Figure 4.1(ii)). Tobramycin monotherapy at 10µg/ml also demonstrated similar potency as ciprofloxacin (Figure 4.1(iii)). 8 µg/ml Azithromycin monotherapy did not exhibit any effect on the PAO1 biofilms (Figure 4.1 (iv)). Carbenicillin monotherapy at 300 µg/ml also demonstrated effective killing ability by targeting the metabolic active population of the biofilms (Figure 4.1 (v)). Similar to the data obtained from our previous study, CHIR-090 monotherapy at 4 µg/ml only targets the metabolically active population of the biofilm, but the dormant portion of the biofilms remains unaffected. (Figure 4.1(vi)).

Next, each antibiotic was combined with CHIR-090 to evaluate the combined killing efficacy against PAO1 biofilms. Interestingly, the combination of 10 µg/ml of ciprofloxacin and 4 µg/ml of CHIR-090 seem to be antagonistic resulting in minimal killing of PAO1 biofilms (Figure 4.1(vii)). Tobramycin-CHIR-090 combination increased killing effect of PAO1 biofilms as compared to monotherapy of the individual antimicrobials (Figure 4.1(viii)). Combination of azithromycin at 8 µg/ml and CHIR-090 at 4 µg/ml did not result in any synergy (Figure 4.1(ix)). Surprisingly, contrary to our MBEC study, combination of carbencilin and CHIR-090 showed synergy by eradicating both metabolically active and dormant population of the biofilms (Figure 4.1 (x)).

Figure 4.1: *In vitro* efficacy of tobramycin, ciprofloxacin, azithromycin, carbencillin and CHIR-090 alone or in combination against 3-day-old PAO1 biofilms in biofilm flow cells. (A) Confocal imaging of the killing effects of DMSO (control) (i), ciprofloxacin at 10 $\mu\text{g/ml}$ (ii), tobramycin at 10 $\mu\text{g/ml}$ (iii), azithromycin at 8 $\mu\text{g/ml}$ (iv), carbencillin at 300 $\mu\text{g/ml}$ (v), CHIR-090 at 4 $\mu\text{g/ml}$ (vi), 10 $\mu\text{g/ml}$ of ciprofloxacin combined with CHIR-090 at 4 $\mu\text{g/ml}$ (vii), 10 $\mu\text{g/ml}$ of tobramycin combined with CHIR-090 at 4 $\mu\text{g/ml}$ (viii), 8 $\mu\text{g/ml}$ of azithromycin combined with CHIR-090 at 4 $\mu\text{g/ml}$ (ix) and 300 $\mu\text{g/ml}$ of carbencillin with CHIR-090 at 4 $\mu\text{g/ml}$ (x) Scale bars 50 μm .

4.2.4 Time-kill assay of PAO1 biofilms with tobramycin and CHIR-090 combination

Given that tobramycin and CHIR-090 combination demonstrated synergy in the different *in-vitro* systems such as 96 wells microplate biofilm assay and flow cell model, we further investigated the killing dynamics of tobramycin and CHIR-090 combination using time-kill assay. In this assay, biofilms were grown on glass beads for 3 days and subjected to 20 hours treatment with the following treatment regime: (i) Control (DMSO), (ii) 4 $\mu\text{g/ml}$ CHIR-090, (iii) 10 $\mu\text{g/ml}$ tobramycin and (iv) 10 $\mu\text{g/ml}$ tobramycin and 4 $\mu\text{g/ml}$ CHIR-090 combination. The initial inoculum was

approximately 10^6 CFU per ml. As expected, the biofilm bacteria count for the control group treated with DMSO remained unaffected throughout the experiment. For CHIR-090 monotherapy, there was a decrease in CFU count by 10^2 folds after 8 hours of treatment. However, CFU increased to 10^5 at the 12th hours and growth was inhibited for next 8 hours. This result was found to be consistent with MBEC and flow cell model results. Similarly for tobramycin monotherapy, CFU of biofilm decreased to approximately 10^2 CFU at the 8th hours. After 16 hours of tobramycin monotherapy, CFU decreased below detectable level, however, biofilms revived to 10^3 CFU at the 20th hour. This suggested that the bactericidal activity of tobramycin might have worn off after long hours of incubation or the presence of “persister cells” might have contributed to the revival of the biofilms. Tobramycin-CHIR-090 combination enabled faster killing of biofilm as bacteria count became undetectable at the 8th hour treatment. Interestingly, there was an increase of the CFU to 10^3 at 12th hour but became undetectable again for the next 8 hours. Overall, the results suggested that tobramycin-CHIR-090 combination enable faster killing of the biofilms and suppressed biofilm re-growth.

Figure 4.3: Time-kill assay of 3 days old PAO1 biofilms. 3 days old biofilms were on 3mm glass beads and treated with i) control (DMSO) ii) CHIR-090 4μg/ml iii) tobramycin 10 μg/ml and iv) tobramycin 10 μg/ml and CHIR-090 4 μg/ml for 20 hours

4.2.5 *In vivo* evaluation of tobramycin and CHIR-090 combination, against PAO1 biofilms grown in a mouse implant model of infection.

A preliminary evaluation of the *in vivo* efficacy of tobramycin and CHIR-090 combination was conducted using a mouse implant biofilm model. Similar bacteria counts were observed from the mice from the control group (treated with DMSO) in both implant and spleen (Figure 4.4). Interestingly, both CHIR-090 and tobramycin monotherapy achieved only minimal killing of biofilms comparable to the control (Figure 4.4). Combination of tobramycin and CHIR-090 resulted in a reduction of 2 log in bacteria counts (Figure 4.4). Bacteria counts ($\sim 10^5$) in the spleen were similar among DMSO, CHIR-090 and tobramycin treated group. Lower bacteria load were observed in the spleen with the tobramycin and CHIR-090 combination.

Figure 4.4: Preliminary *in vivo* evaluation of tobramycin (10 µg/ml) and CHIR-090 (4 µg/ml) in combination using a mouse implant model of PAO1 biofilm. Credit to Dr. Joey Yam and Mrs. Mary Salido for performing the *in vivo* mouse implant experiment

4.4 Discussion

Combinational antimicrobial therapy is a promising strategy to enhance treatment efficacy and minimize the probability of resistance development. [241] Given that there is no specific therapeutic strategy that targets biofilms, combination therapy continues to be one of the effective therapeutic methods to increase the likelihood of faster clearance of biofilm infection and hasten the recovery process of the patients. [242]

In our previous study, the results obtained from both *in vitro* and *in vivo* tests demonstrated that colistin and CHIR-090 is an effective combination against PAO1 biofilms and non-susceptible colistin clinical strains. However, colistin is often used in

combination therapy as the last-resort to persistent infections due to nephrotoxicity implications. [236, 237] Patients with impaired renal functions might not be suitable for colistin treatment. [235] In addition, due to the extensive use of colistin, bacterial strains have begun to develop colistin resistance in clinical settings. [204-207] Therefore, there is a need to study other probable combinations of CHIR-090 with other classes of antibiotics and characterize their *in vitro* and *in vivo* anti-biofilm activity against *P.aeruginosa* biofilms.

Combination of four classes of antibiotics with antipseudomonal LpxC inhibitor, CHIR-090 was examined in this study to evaluate their possible synergistic anti-biofilms activity. Four antimicrobials (ciprofloxacin, tobramycin, azithromycin and carbenicillin) were selected because they are commonly used to treat *P.aeruginosa* biofilms infections. Furthermore, combination therapy among these antibiotics had been extensively studied and evaluated for their potency against *P. aeruginosa* biofilms. [66, 85, 86, 238-240] To assess the efficacy of these selected antibiotics in combination with CHIR-090, checkerboard assay was used to calculate the fractional inhibitory concentration, Σ FIC and determined each combination's MIC and MBEC values. Out of the four antibiotics combination, only tobramycin-CHIR-090 combination displayed synergy with a Σ FIC value of 0.5 and 0.3125 for MIC and MBEC, respectively. Other three antibiotics did not exhibit any interaction with CHIR-090 against both planktonic and biofilms.

To gain a better understanding how each combination targets the biofilm; the individual combinations were tested in a flow cells system where the condition mimics closely that

of the human body. Consistent with our data, CHIR-090 monotherapy only targets the metabolically active layers of the biofilms. Similarly, ciprofloxacin, tobramycin and carbenicillin monotherapy affected the metabolically active part of the biofilms and these observations were consistent with the previous studies. [85] Lastly, azithromycin monotherapy did not exert any bactericidal effect on the biofilms. Combination of the different antibiotics with CHIR-090 yielded very interesting results. In line with the static system data, there was an increase in the killing of the PAO1 biofilms with tobramycin-CHIR-090 combination. The synergy mechanism might be due to the disruption of the LPS by CHIR-090 and lead to the uptake of tobramycin. Previous studies found that disruption of genes involved in lipid biosynthesis (*faoA* and *lptA*) increased *P. aeruginosa* susceptibility to tobramycin. [178] Once tobramycin penetrates the cells, it will cause ribosome disruption and lead to production of mistranslated membrane-damaging polypeptides. [243] This might in turn increase the uptake of CHIR-090 and tobramycin. Another possible mechanism of synergy is suppression of the expression of efflux pump. *In vitro* transcriptomic analysis of tobramycin treated *P.aeruginosa* biofilms showed that the expression of multidrug resistance repressor *MexR* and efflux transporter *MexB* were upregulated and downregulated, respectively. [244] CHIR-090 is a substrate of *MexAB-OrpM* while *MexR* is a repressor of *MexAB* operon. [153, 170] We hypothesized that tobramycin treatment may decrease the expression of *MexAB-OrpM* efflux pump and in turn increased the susceptibility of the biofilms to CHIR-090.

Interestingly, based on the confocal microscopy images, ciprofloxacin-CHIR-090

combination seemed to be antagonistic as the biofilms treated with the combination was unaffected. We speculated that the antagonistic effect might due to the overexpression of the efflux pump such as *MexAB-OprM*, *MexCD-OprJ* and *MexEF-OprN*. Studies have shown that CHIR-090 and ciprofloxacin are the substrate for these efflux pumps. [124, 153] There is a possibility that the combination of CHIR-090 and ciprofloxacin may have simultaneously induced and increased expression of the multicomponent efflux pumps, enhancing antibiotic resistance. [245] Alternatively, the antagonistic effect could be due to drug-drug interaction between CHIR-090 and ciprofloxacin. Further pharmacological experiments are needed to verify this hypothesis.

For azithromycin and CHIR-090 combination, the confocal results were consistent with that of the static systems with no synergy in the combination. However, this combination should be further investigated in *in vivo* experiment to determine its actual efficacy as azithromycin is known to have anti-virulence, anti-quorum sensing properties, retarded biofilms growth, and is demonstrated to improve the clinical outcome of cystic fibrosis patients. [66, 238, 239] Surprisingly, the combination of carbenicillin and CHIR-090 applied in a flow cell model demonstrated a different observation from the static biofilms. The combination seemed to be capable of targeting both metabolically active and dormant part of the biofilms. There was an increase in killing of the biofilms as propidium iodide (PI) signal intensified. In the previous experiments, CHIR-090 and carbenicillin monotherapy were shown only targeting the metabolically active layer of the biofilms, hence it came as a surprise to observe such killing pattern of the biofilms. The mechanism of this synergy is currently unknown and remains to be elucidated.

Given that tobramycin-CHIR-090 combination demonstrated synergy in the checkerboard assay and flow cells system, the combination was selected for time-kill assay to better understand the killing dynamics. The result showed that the combination enable faster killing of the biofilms as the bacteria counts became undetectable after 8 hours of treatment and also suppressed of the regrowth of the biofilms. This indicates that the combination is likely to be more efficient in the killing of biofilms, shorten both treatment period and patient recovery time. *In vivo* mouse biofilm implant experiment supported the *in vitro* observations, with a combination of tobramycin and CHIR-090 being more effective than their respective monotherapy. However, the single-dose regimen used in the experiment could not achieve a total eradication of the biofilms. Administration of repeated dosages over a course of 5 to 7 days would provide us with a better prognosis of the therapeutic outcomes of this combined treatment. Finally, contrary to the colistin-CHIR-090 combination, the combined treatment of tobramycin and CHIR-090 did not restrict the spread of the bacteria to the spleen. More studies are warranted to study clinically relevant dosages based on the area under the concentration-time curve/MIC parameter to further the therapeutic potential of tobramycin-CHIR-090 combination.

Finally, for the first time, our study evaluated the *in vitro* antimicrobial efficacies of CHIR-090 combined with different classes of antibiotics such as fluoroquinolones, aminoglycosides, β -lactams and macrolides. Out of the four combinations, only tobramycin and CHIR-090 was found to be synergistic in static system, flow cell and

mouse implant model. This data provided evidence for alternative combination beside colistin-CHIR-090 combination. More importantly, future work should consider combining CHIR-090 with other antibiotics within the four classes such as imipenem, clarithromycin, tigecycline and many others that have been evaluated to effective in combination therapy against *P. aeruginosa* biofilms. [246-249]

Chapter 5: Conclusion and Future Plan

5.1 Conclusion

Biofilms are multicellular bacterial communities that formed on a surface and encased in an extracellular polymeric substance (EPS) made up of eDNA, proteins and exopolysaccharide. Biofilms are responsible for causing biofilms-associated infections and are more difficult to treat due to increased antibiotic resistance. Furthermore, biofilms are highly prevalent and associated with most of microbial infections. Due to the lack of specific anti-biofilm treatment, combination antimicrobial therapy remains one of the most effective methods to eradicate biofilms infection. In the recent decade, a new class of antimicrobial that targets the LpxC enzyme was discovered. Among the LpxC inhibitors discovered, CHIR-090 was the only and most effective compound against *P. aeruginosa* with efficacy comparable to ciprofloxacin and tobramycin. However, the possible stress responses and resistance mechanisms of PAO1 against CHIR-090 have yet to be fully understood. Also, the anti-biofilm activity of CHIR-090 and its combination with other class of antimicrobial has yet been investigated.

In the first chapter of this thesis, a preliminary study was conducted to examine the stress responses of planktonic PAO1 treated with sub-inhibitory concentration of CHIR-090 using RNA-sequencing transcriptomic analyses. A total of 109 genes were affected with 76 genes upregulated and 30 genes downregulated. Among the upregulated genes, three gene clusters associated with antibiotic resistance to other class of antibiotics were identified. They are the *faoAB* operon with its *psrA* transcription regulator involved in

fatty acid synthesis, *mexCD-OrpJ* that encodes for RND efflux pump and *phoPQ-oprH* two components transcription regulator. Six mutations were identified in the preliminary genome analyses of PAO1 CHIR-090 resistant strain, 090-TJH. The most common mutated gene identified among the three resistant populations is *mexR*, a multidrug transcriptional repressor of the *mexAB-oprM* efflux pump. The analysis also identified the mutation of three genes associated with cell membrane or fatty acid synthesis; *murB*, *pyrG* and *fabF*. Two genes associated with flagellar assembly or motility and biofilm formation, *fliF* and *morA* were also mutated. These two different sets of analysis revealed a common trend that the general resistance mechanism against CHIR-090 usually involved the efflux pump and alteration of the fatty acid/cell membrane synthesis.

In the following two chapters of this thesis, the anti-biofilm activity of CHIR-090 alone, and combined with other classes of antimicrobials against PAO1 biofilms was evaluated using *in vitro* and *in vivo* methods.

The first combination evaluated was CHIR-090 and colistin. Since both CHIR-090 and colistin targets LPS of Gram-negative bacteria, we hypothesized that the combination could be synergistic through a self-promoted uptake mechanism. In this study, four *P. aeruginosa* isolates with varying colistin susceptibility were chosen for evaluation. Tested isolates of *P. aeruginosa* exhibited MIC values ranging from 1 to 64 µg/ml and 0.0625 to 0.5 µg/ml for colistin and CHIR-090, respectively. Against 24 hours static biofilms, MBEC values ranged from 256 to 512 µg/ml and 8 to >128 µg/ml for colistin

and CHIR-090, respectively. Interestingly, sub-inhibitory concentrations of CHIR-090 eradicated subpopulations of *P. aeruginosa* with highest colistin MIC values. Combination of colistin and CHIR-090 at sub-inhibitory concentrations demonstrated synergistic activity, both *in vivo* and *in vitro*, and prevented the formation of colistin tolerant sub-populations in *in vitro* biofilms. This study highlighted *in vivo* and *in vitro* synergistic activity of colistin and CHIR-090 combination against both colistin-susceptible and non-susceptible *P. aeruginosa* biofilms.

In the last chapter, we expanded the antimicrobial combination options of the LpxC, CHIR-090 by evaluating the anti-biofilm activity of CHIR-090 combined with four different classes of antimicrobials that commonly used to treat *P.aeruginosa* biofilms. These four antibiotics are carbencillin (β -lactams), ciprofloxacin (fluoroquinolones), tobramycin (aminoglycosides) and azithromycin (macrolide). Only tobramycin and CHIR-090 showed *in vitro* synergistic activity against planktonic PAO1 and biofilm, with a Σ FIC value of 0.5 (0.25 μ g/ml + 0.25 μ g/ml) for MIC and a Σ FIC value of 0.3125 (4 μ g/ml + 16 μ g/ml) for MBEC. No interaction was observed with other combinations. Consistent with MIC and MBEC results, 10 μ g/ml of tobramycin and 4 μ g/ml of CHIR-090 combination showed synergy against 3 days PAO1 biofilms under flow cell condition. Interestingly, combination of 10 μ g/ml ciprofloxacin and 4 μ g/ml CHIR-090 was antagonistic under the flow cell condition as there was minimal killing of PAO1 biofilms. Contrary to the MIC and MBEC result, combination of 300 μ g/ml of carbencillin and 4 μ g/ml of CHIR-090 increased the killing effect of the biofilms comparing to their individual monotherapy. Time-kill assay demonstrated that

tobramycin-CHIR-090 combination enable faster killing of the biofilms and also suppressed the re-growth of biofilms. Preliminary study of tobramycin-CHIR-090 combination in *in vivo* biofilms implant mouse model showed that the combination reduced at least 2 log of bacteria load but was unable to control the spread of the bacteria to the spleen. The observations in this study demonstrated the *in vitro* and *in vivo* synergistic activity of the tobramycin-CHIR-090 combination against PAO1 biofilms.

Overall, the results from these three studies demonstrated that the possible resistance mechanisms and stress responses of planktonic PAO1 against CHIR-090 and characterized the *in vitro* and *in vivo* of the anti-biofilm activity of CHIR-090 alone, in combination with colistin and other class of antimicrobials. Ultimately, this study provided evidences for therapeutic potential of LpxC inhibitors against *P.aeruginosa* biofilms and combination therapy options to overcome antimicrobial resistance.

5.2 Future Plan

The preliminary study of the stress responses and resistance mechanisms of planktonic PAO1 treated with CHIR-090 using transcriptomic and genome analyses gave us an overview of the genes that are associated with antibiotic resistance. However, validation studies are required to confirm these identified genes and these can be achieved through RT-PCR and gene knockout experiments. Given that biofilm is the prevalent mode of infection, the transcriptomic analysis should also be extended to study biofilm samples treated with CHIR-090. This information will provide us a better understanding on the molecular resistance mechanism in biofilms during CHIR-090 treatment.

Given that combinations of CHIR-090 with colistin and tobramycin had shown to be synergistic, it would be insightful to elucidate the possible mechanism for synergy. The synergy mechanism can be elucidated by transcriptomic analysis of the biofilm sample treated by the individual monotherapy and combined therapy. By comparing the expression profiles obtained from the analysis, we might be able to identify the synergy pathway that might be affected in the combined treatment but not in the monotherapy. Furthermore, combination of CHIR-090 and tobramycin should be tested on clinical isolate so that we would have better information on how this potency of combination in the clinical setting.

The different classes of antibiotics tested in this study are not exhaustive. Many other antibiotics within the same class have yet to be tested in combination with CHIR-090. Hence, it would be interesting to study the anti-biofilm activity of other antibiotics with CHIR-090 against PAO1 biofilms. Also, the combined treatment should be extended to other Gram-negative bacteria biofilms. This will determine whether such combination has a broad-spectrum in the eradication of different Gram-negative species.

Finally, many CHIR-090 derivatives have been synthesized since the discovery of CHIR-090. While our study showed that CHIR-090 alone does not eradicate PAO1 biofilms, it would still be feasible to examine the potency of other CHIR-090 derivatives against PAO1 biofilms. It would also be interesting to investigate the combination of CHIR-090 derivatives with other classes of antimicrobials against PAO1 or other Gram-

negative biofilms. This would tremendously increase the therapeutic options against biofilms and ultimately, benefit patients in their recovery.

Chapter 6: Reference

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Chapter 7: Appendix

7.1 Transcriptiomic data

Genes upregulated in PAO1 treated with 0.5 ug/ml CHIR-090			
Gene	Protein	Fold Change	FDRcorrected pvalue
pntAB	hypothetical protein	2.001149627	1.29E-05
algU	sigma factor AlgU	2.005774466	2.42E-07
PA1188	hypothetical protein	2.034804773	4.02E-05
zwf	glucose-6-phosphate 1-dehydrogenase	2.042539157	7.88E-12
lipA	lactonizing lipase precursor	2.043278873	2.52E-05
aceA	hypothetical protein	2.059104297	1.42E-11
PA1517	hypothetical protein	2.077820315	2.05E-07
PA1289	hypothetical protein	2.078820073	1.37E-05
PA1029	hypothetical protein	2.08866915	1.29E-06
PA1673	hypothetical protein	2.092705791	4.27E-08
phoQ	two-component sensor PhoQ	2.096974785	1.01E-08
PA4620	hypothetical protein	2.109870517	7.37E-06
fadD1	long-chain-fatty-acid—CoA ligase	2.126967384	8.76E-11
etfB	electron transfer flavoprotein beta-subunit	2.137854065	1.07E-15
PA2482	hypothetical protein	2.140763044	0.000225289
PA3973	hypothetical protein	2.166985039	2.71E-05
PA4917	hypothetical protein	2.190166634	1.48E-08
glpT	glycerol-3-phosphate transporter	2.191990754	4.18E-10

PA4596	hypothetical protein	2.250462059	6.10E-10
phoP	two-component response regulator PhoP	2.263003726	1.04E-12
PA1604	hypothetical protein	2.275373674	3.75E-11
PA1622	hypothetical protein	2.333243271	1.25E-08
pntB	pyridine nucleotide transhydrogenase, beta subunit	2.347452433	7.29E-18
pgl	6-phosphogluconolactonase	2.378859729	8.20E-06
PA5157	hypothetical protein	2.409743056	1.25E-07
PA2814	hypothetical protein	2.447129597	8.77E-10
PA4824	hypothetical protein	2.447644552	0.000502257
PA0830	hypothetical protein	2.447660458	7.12E-11
PA4822	hypothetical protein	2.450245845	0.000249229
etfA	electron transfer flavoprotein alpha-subunit	2.453858752	2.14E-28
PA1196	hypothetical protein	2.469170328	3.00E-13
PA2665	hypothetical protein	2.471215729	1.18E-11
PA5435	hypothetical protein	2.471295908	1.77E-27
PA0200	hypothetical protein	2.491551562	5.66E-16
PA5436	hypothetical protein	2.493347286	3.34E-25
glpK	glycerol kinase	2.497068075	3.46E-15
PA1429	hypothetical protein	2.507252337	1.35E-14
PA5446	hypothetical protein	2.534958114	5.91E-16
PA1187	hypothetical protein	2.577147215	1.18E-10
PA4821	hypothetical protein	2.647927138	7.12E-11

PA3676	hypothetical protein	2.649669614	2.35E-19
pncB1	nicotinate phosphoribosyltransferase	2.682747274	1.74E-21
arcD	arginine/ornithine antiporter	2.795661969	2.60E-22
PA4610	hypothetical protein	2.858511062	1.95E-08
PA1283	hypothetical protein	3.039786993	1.92E-20
glpD	glycerol-3-phosphate dehydrogenase	3.04356776	5.80E-14
PA0529	hypothetical protein	3.19496897	4.79E-14
PA1542	hypothetical protein	3.204451967	4.10E-14
PA2136	hypothetical protein	3.218701392	2.71E-08
mgtA	“Mg(2+) transport ATPase, P-type 2”	3.220901613	3.31E-12
PA2759	hypothetical protein	3.330186823	1.74E-21
PA1830	hypothetical protein	3.442202579	1.74E-21
narK1	nitrite extrusion protein 1	3.546591597	1.82E-16
glpF	glycerol uptake facilitator protein	3.554280792	2.23E-12
oprH	PhoP/Q and low Mg ²⁺ inducible outer membrane protein H1 precursor	3.737530486	4.09E-32
PA3677	hypothetical protein	3.900149237	1.83E-32
PA3925	hypothetical protein	3.975809987	3.57E-60
PA0276	hypothetical protein	4.106463635	9.73E-13
PA3971	hypothetical protein	4.19589427	2.59E-15
PA4435	hypothetical protein	4.234509173	4.96E-39
PA4918	hypothetical protein	4.239890658	1.44E-45
PA1343	hypothetical protein	4.411471641	5.25E-17

mexC	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein MexC precursor	4.544462437	3.41E-48
PA1737	hypothetical protein	4.67694951	7.79E-52
PA2753	hypothetical protein	4.706808117	4.48E-52
PA1282	hypothetical protein	5.110248572	2.63E-23
PA1736	hypothetical protein	5.136565835	1.47E-52
PA3972	hypothetical protein	5.274145855	3.90E-63
psrA	transcriptional regulator PsrA	5.307320445	6.21E-27
PA2953	electron transfer flavoprotein-ubiquinone oxidoreductase	5.530776407	7.24E-52
mexD	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexD	5.679779806	8.17E-77
oprJ	Multidrug efflux outer membrane protein OprJ precursor	6.151583119	2.62E-46
PA0508	hypothetical protein	6.833150172	2.04E-92
faoA	fatty-acid oxidation complex alpha-subunit	7.333121321	6.05E-66
foaB	fatty-acid oxidation complex beta-subunit	10.50841372	9.94E-127
PA0506	hypothetical protein	13.05591884	2.46E-129

Table 7.1.1: List of upregulated genes in *P.aeruginosa* PAO1 treat with CHIR-090.

Selection criteria were set as fold change ≥ 2 and p-value ≤ 0.05 , n = 3. Gene names, protein name, fold-change and corrected p-value are shown. Credit to Dr. Gurjeet Singh for the transcriptomic analysis.

Genes downregulated in PAO1 treated with 0.5 ug/ml CHIR-090			
Gene	Protein	Fold Change	FDRcorrected pvalue
cysD	ATP sulfurylase small subunit	-3.757236367	1.17E-27
PA0284	hypothetical protein	-3.450121154	3.19E-23
sbp	sulfate-binding protein precursor	-3.435883584	3.34E-25
PA2786	hypothetical protein	-3.228403062	1.48E-08
PA3450	hypothetical protein	-3.133754292	5.99E-25
PA4638	hypothetical protein	-3.114478159	8.93E-15
PA4195	hypothetical protein	-2.926660856	1.37E-12
PA2062	hypothetical protein	-2.859470634	3.80E-18
PA2083	hypothetical protein	-2.662078479	1.70E-13
PA2204	hypothetical protein	-2.592186815	2.64E-18
PA2203	hypothetical protein	-2.571195615	3.38E-06
cpdA	hypothetical protein	-2.503546781	1.10E-11
cysT	sulfate transport protein CysT	-2.470886332	1.15E-10
PA0104	hypothetical protein	-2.421220955	3.37E-14
PA3931	hypothetical protein	-2.418168031	7.70E-09
phzA2	hypothetical protein	-2.403588214	3.95E-07
cysH	3'-phosphoadenosine-5'-phosphosulfate reductase	-2.385646292	3.22E-11
PA2365	hypothetical protein	-2.332861343	1.37E-12

PA3443	hypothetical protein	-2.28996937	1.95E-05
PA3842	hypothetical protein	-2.216781352	5.81E-05
PA2367	hypothetical protein	-2.197169837	1.02E-11
cysA	sulfate transport protein CysA	-2.186796809	2.00E-13
PA3445	hypothetical protein	-2.170559947	3.74E-09
cysW	sulfate transport protein CysW	-2.108363357	1.74E-08
gcdH	glutaryl-CoA dehydrogenase	-2.086265728	1.10E-11
PA3956	hypothetical protein	-2.078806807	5.36E-05
PA3938	hypothetical protein	-2.048007622	1.61E-07
aspA	aspartate ammonia-lyase	-2.018660009	1.38E-16
PA0201	hypothetical protein	-2.014693426	1.60E-07
PA2366	hypothetical protein	-2.012315609	3.99E-14

Table 7.1.2: List of downregulated genes in *P.aeruginosa* PAO1 treat with CHIR-090.

Selection criteria were set as fold change ≥ -2 and p-value ≤ 0.05 , n = 3. Gene names, protein functions, fold-change and corrected p-value are shown. Credit to Dr. Gurjeet Singh for the transcriptomic analysis.

7.2 Genomic analysis data

Population: TJH01

Region	Type	Reference	Allele	Zygosity	Coverage	Frequency	Coding region change	Amino acid change	Non-synonymous	NC_002516 (Gene)	product (NC_002516 (CDS))
471541	SNV	C	A	Homozygous	606	100	IP_249115.1:c.209G>T	NP_249115.1:p.Arg70Leu	Yes	mexR	multidrug resistance operon repressor MexR
721661^721662	Insertion	-	C	Heterozygous	27	59.25925926			-		
721661^721662	Insertion	-	-	Heterozygous	27	40.74074074			-		
794456	SNV	G	C	Heterozygous	897	35.78595318			-		
794456	SNV	G	G	Heterozygous	897	64.1025641			-		
3336504	SNV	T	C	Homozygous	447	99.77628635	IP_251667.1:c.728A>G	NP_251667.1:p.Asn243Ser	Yes	murB	UDP-N-acetylenolpyruvoylglucosamine reductase
5159261	SNV	G	A	Homozygous	523	99.61759082	IP_253291.1:c.2894G>A	NP_253291.1:p.Ser965Asn	Yes	morA	motility regulator

Table 7.2.1: List of mutations found in CHIR-090 resistant population-TJH01. Credit to Dr. Ding Yichen for the genomic analysis

Population: TJH02

Region	Type	Reference	Allele	Zygosity	Coverage	Frequency	Coding region change	Amino acid change	Non-synonymous	NC_002516 (Gene)	product (NC_002516 (CDS))
471669^471670	Insertion	-	A	Homozygous	454	99.559471	NP_249115.1:c.80_81insT	NP_249115.1:p.Glu27fs	Yes	mexR	multidrug resistance operon repressor MexR
721661^721662	Insertion	-	C	Homozygous	32	78.125			-		
1192551.1192552	Deletion	CG	-	Homozygous	492	78.861788	NP_249792.1:c.147_148del CG	NP_249792.1:p.Ala50fs	Yes	fliF	flagellar MS-ring protein
3336547	SNV	G	A	Homozygous	515	99.611650	NP_251667.1:c.685C>T	NP_251667.1:p.Pro229Ser	Yes	murB	UDP-N-acetylenolpyruvoylglucosamine reductase

Table 7.2.1: List of mutations found in CHIR-090 resistant population-TJH02. Credit to Dr. Ding Yichen for the genomic analysis

Population: TJH03

Region	Type	Reference	Allele	Zygoty	Coverage	Frequency	Coding region change	Amino acid change	Non-synonymous	NC_002516 (Gene)	product (NC_002516 (CDS))
471639^471640	Insertion	-	G	Homozygous	545	92.29357798	NP_249115.1:c.110_111insC	NP_249115.1:p.Pro38fs	Yes	mexR	multidrug resistance operon repressor MexR
721661^721662	Insertion	-	C	Heterozygous	33	45.454545			-		
721661^721662	Insertion	-	-	Heterozygous	33	54.545454			-		
794456	SNV	G	C	Heterozygous	986	35.59837728			-		
794456	SNV	G	G	Heterozygous	986	64.30020284			-		
3324007	SNV	G	A	Heterozygous	429	48.71794872	NP_251655.1:c.812C>T	NP_251655.1:p.Thr271Ile	Yes	fabF1	3-oxoacyl-ACP synthase
3324007	SNV	G	G	Heterozygous	429	51.28205128			No	fabF1	3-oxoacyl-ACP synthase
4071897	SNV	G	T	Heterozygous	564	46.63120567	NP_252327.1:c.592C>A	NP_252327.1:p.Arg198Ser	Yes	pyrG	CTP synthetase
4071897	SNV	G	G	Heterozygous	564	53.36879433			No	pyrG	CTP synthetase

Table 7.2.3: List of mutations found in CHIR-090 resistant population-TJH03. Credit to Dr. Ding Yichen for the genomic analysis